MICROBIAL EVALUATION OF SELECTED PRODUCE PRE- AND POST-PACK-HOUSE AND AT THE FORMAL RETAIL POINT-OF-SALE

by

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DECLARATION

By submitting this thesis electronically, I declare that the entirety of the work contained therein is my own, original work, that I am the sole author thereof (save to the extent explicitly otherwise stated), that reproduction and publication thereof by Stellenbosch University will not infringe any third party rights and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

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ABSTRACT

Fresh produce consumption is important to humans as it provides important nutrients and other compounds that promote good health. However, consumption of contaminated produce can be detrimental to human health. Outbreaks linked to fresh produce consumption have been reported globally, with *Enterobacteriaceae* members such as *Escherichia coli* and *Salmonella* being the most frequently implicated bacteria. Fresh produce isolates carrying the extended spectrum β-lactamase (ESBL) producing *Enterobacteriaceae* has been reported. These organisms can resist the action of penicillin and the broad-spectrum cephalosporins, and they are also resistant to other antimicrobials. This is such a concern because fresh produce is eaten raw and these organisms are not inactivated before consumption. To be able to control the spread of contaminations and antimicrobial resistance along the fresh produce production chain, it is essential to know the microbiological quality of fresh produce at different stages of production.

The aim of this study was to determine the changes in the microbiological quality of fresh produce pre- and post-pack-house processing and at the formal point-of-sale, in order to identify potential contamination points along the supply chain. Different fresh produce types: broccoli coleslaw (broccoli stems, carrots and cabbage) and lettuce samples were collected at different processing points within a pack-house situated in Phillippi, Western Cape, South Africa. Some pack-house samples (mixed coleslaw bags and lettuce pre-packs) were also collected from retail outlets. All samples were tested for microbial indicators (*Enterobacteriaceae*, coliforms and *E. coli*), *Salmonella* and Shiga-toxin producing *E. coli* (STEC). Produce samples were also screened for ESBL-producing *Enterobacteriaceae*.

The untreated/unprocessed samples had high microbial counts which were then reduced to significantly lower levels after peeling and washing in a chlorine (150-200 ppm) solution. An increase in microbial counts to levels significantly higher than on the treated samples was observed in shredded samples and bagged mix coleslaw samples. Mixed coleslaw bags sampled from the retailer two days after packaging also had significantly higher microbial levels than mixed coleslaw from the same batch sampled at the pack-house directly after packaging. Lettuce samples have indicated a gradual decrease on microbial levels throughout, and the lowest reduction was detected on pillow-packs samples. Throughout the study, no *Salmonella* or STEC were detected.

Fifty isolates were identified as *Enterobacteriaceae* with MALDI-TOF, of which 22% were confirmed as ESBL producers according to the EUCAST disk diffusion method (2017b). All 50 *Enterobacteriaceae* were also subjected to genotypic confirmation, and seven of them were carrying the ESBL genes: $bla_{\text{CTX-M}}$ and $bla_{\text{TEM.}}$ *Enterobacter cloacae* and

Klebsiella oxytoca isolates were found carrying bla_{CTX-M} and bla_{TEM}, and a single bla_{TEM} was found on an *E. coli* isolate. All 50 *Enterobacteriaceae* were also tested for resistance against ampicillin, gentamicin, tetracycline, ciprofloxacin, and chloramphenicol. Five of the 50 tested isolates were found to be multidrug resistant. Fresh produce is eaten raw without thermal treatment to deactivate these organisms carrying ESBL genes. Through ingesting of this produce the ESBL genes could be transferred to the intestinal microorganisms and will confer resistance to important antimicrobials. This study investigated the microbiological quality of fresh produce sold in the Western Cape and has also identified shredding and packaging as potential contamination points. Given favourable conditions, microorganisms may grow on stored fresh produce over time.

OPSOMMING

Die verbruik van vars produkte is vir mense belangrik aangesien dit belangrike voedingstowwe en ander verbindings bied wat goeie gesondheid bevorder. Die verbruik van gekontamineerde produkte kan egter die gesondheid van mense benadeel. Daar is wêreldwyd sprake van uitbrake wat gekoppel is aan die verbruik van vars produkte, met lede van *Enterobacteriaceae* soos *Escherichia coli* en *Salmonella* as die bakterieë wat die meeste geïmpliseer word. Vars produkte-isolate wat die *Enterobacteriaceae* bevat wat verlengde spektrum ß-laktamase (ESBL) produseer, is aangemeld. Hierdie organismes kan die werking van penisillien en die breë-spektrum kefalosporiene weerstaan, en is ook bestand teen ander antimikrobiese middels. Dit is so kommerwekkend omdat vars produkte rou geëet word en hierdie organismes nie voor verbruik geïnaktiveer word nie. Om die verspreiding van kontaminasie en antimikrobiese weerstandbiedendheid in die vars produk produksieketting te kan beheer, is dit noodsaaklik om die mikrobiologiese kwaliteit van vars produkte in verskillende produksiestadia te weet.

Die doel van hierdie studie was om die veranderinge in die mikrobiologiese gehalte van die voor- en na-pakhuisverwerking van vars produkte en by die formele verkooppunt te bepaal, ten einde potensiële kontaminasiepunte rondom die produksieketting te identifiseer. Verskillende soorte vars produkte: broccoli koolslaai (broccoli-stingels, wortels en kool); en blaarslaai-monsters is by verskillende verwerkingspunte in 'n pakhuis in Phillippi, Wes-Kaap, Suid-Afrika, versamel. Sommige pakhuismonsters (gemengde koolslaai-sakkies en blaarslaai-pakkies) is ook by kleinhandelswinkels versamel. Al die monsters is getoets vir mikrobiese indikators (*Enterobacteriaceae*, kolivorme en *E. coli*), *Salmonella* en Shiga-toksien-produserende *E. coli* (STEC). Vars produk monsters is ook getoets vir ESBL-produserende *Enterobacteriaceae*.

Die onbehandelde / onbewerkte monsters het 'n hoë mikrobiese telling wat dan na afskil en was in 'n chlooroplossing (150-200 dpm) tot aansienlik laer vlakke verminder is. 'n Toename in mikrobiese tellings tot vlakke wat beduidend hoër is as by die behandelde monsters, is waargeneem in gesnipperde monsters en verpakte gemengde koolslaai monsters. Gemengde koolslaai-sakke wat twee dae na verpakking by die kleinhandelaar gemonster is, het ook beduidend hoër mikrobiese vlakke as gemengde koolslaai uit dieselfde lot wat direk na verpakking by die pakhuis geneem is. Blaarslaai-monsters het deurgaans 'n geleidelike afname van mikrobiese vlakke aangedui, en die laagste vermindering is waargeneem by opgeblaste. Gedurende die studie is geen *Salmonella* of STEC opgespoor nie.

Vyftig isolate is met MALDI-TOF geïdentifiseer as *Enterobacteriaceae*, waarvan 22% volgens die EUCAST-metode (2017b) as ESBL-produsente bevestig is. Al 50 *Enterobacteriaceae* is ook aan genotipiese bevestiging onderwerp, en sewe van hulle het die ESBL-geen gedra: bla_{CTX-M} en bla_{TEM}. *Enterobacter cloacae* en *Klebsiella oxytoca* isolate is gevind met bla_{CTX-M} en bla_{TEM}, en 'n enkele bla_{TEM} is in 'n *E. coli* isolaat gevind. Al 50 *Enterobacteriaceae* is ook getoets vir weerstandbiedendheid teen ampisillien, gentamisien tetrasiklien, siprofloksasien en chlooramfenikol. Daar is gevind dat vyf van die 50 getoetste isolate bestand was teen veelvuldige middels. Vars produkte word rou geëet sonder termiese behandeling om hierdie organismes wat ESBL-gene dra te deaktiveer. Deur die inname van hierdie produkte kan die ESBL-gene na die derm-mikroörganismes oorgedra word en kan dit weerstandbiedendheid teen belangrike antimikrobiese middels oordra. Hierdie studie het die mikrobiologiese gehalte van vars produkte wat in die Wes-Kaap verkoop word, ondersoek en het ook versnippering en verpakking as moontlike besmettingspunte geïdentifiseer. Gegewe gunstige toestande, kan mikroörganismes mettertyd op gebergde vars produkte groei.

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TABLE OF CONTENT

This thesis is presented in the format prescribed by the Department of Food Science at Stellenbosch University. The structure is in the form of one or more research chapters (papers prepared for publication) and is prefaced by an introduction chapter with the study objectives, followed by a literature review chapter and culminating with a chapter for elaborating a general discussion and conclusion. Language, style and referencing format used are in accordance with the requirements of the *International Journal of Food Science and Technology*. This thesis represents a compilation of manuscripts where each chapter is an individual entity and some repetition between chapters has, therefore, been unavoidable.

ABBREVIATIONS

AMR	Antimicrobial resistance
ANOVA	Analysis of Variance
ATCC	America Type Culture Collection
BPW	Buffered Peptone Water
CDC	Centres for Disease Control and Prevention
CFU	Colony forming units
CLSI	Clinical and Laboratory Standards Institute
DAEC	Diffusely adherent *Escherichia coli*
DBPs	Disinfection by-products
DNA	Deoxyribonucleic acid
DoH	Department of Health
DWA	Department of Water Affairs
EAEC	Enteroaggregative *Escherichia coli*
EC	European Commission
EC-broth	*Escherichia coli* broth
EE-broth	*Enterobacteriaceae* enrichment broth
EFSA	European Food Safety Authority
EHEC	Enterohaemorrhagic *Escherichia coli*
EIEC	Enteroinvasive *Escherichia coli*
EPEC	Enteropathogenic *Escherichia coli*
ESBL	Extended Spectrum beta-lactamase
ETEC	Enterotoxigenic *Escherichia coli*
EUCAST	European Committee on Antimicrobial Susceptibility Testing
FAO	Food and Agricultural Organisation
HC	Hemorrhagic colitis

HUS	Haemolytic-uremic Syndrome
LEMB	Levine's Eosine Methylene-Blue
MALDI-TOF	Matrix-Assisted Laser Desorption/Ionisation-Time of Flight
NICD	National Institute for Communicable Disease
NSW	New South Wales
OD	Optimal Density
PCR	Polymerase Chain Reaction
RPM	Revolutions per minute
RVS	Rappaport-Vassiliadis Soya
SANS	South African National Standard
SP	Sampling Points
STEC	Shiga toxin producing *Escherichia coli*
Stx	Shiga toxin
TSB	Tryptone Soy Broth
VRBG	Violet Red Bile Glucose
WHO	World Health Organisation
XLD	Xylose Lysine Deoxycholate

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CHAPTER 1

INTRODUCTION

Fresh produce have gained popularity globally due to their nutritional, health, and economical benefits (Johnston *et al.*, 2006; Luo *et al.*, 2018). They provide humans with vitamins, minerals and phytochemicals, which are essential in the fight against cancer and cardiovascular diseases (Schreiner & Huyskens-Keil, 2006; Septembre-malaterre *et al.*, 2018). Many people in developed and developing countries have become aware of these benefits, hence, the consumption of fresh produce has increased globally (Jung *et al.*, 2014). However, the consumption of contaminated fresh produce is linked with bacterial infections and deaths (Wadamori *et al.*, 2017). Fresh produce was identified as a transmission vehicle of human pathogens to consumers. A number of food-borne outbreaks associated with fresh produce consumption have been reported globally (Jung *et al.*, 2014; Wadamori *et al.*, 2017; Murray *et al.*, 2018). Shiga toxin producing *Escherichia coli* (STEC), *Salmonella* and *Listeria monocytogenes* have been the leading bacterial pathogens implicated in food-borne outbreaks associated with fresh produce (Murray *et al.*, 2018). Vegetables mostly implicated in the food-borne outbreaks are cabbages/salads, pre-packaged leafy greens, tomatoes, lettuce, spinach, onions, berries and seed sprouts (FAO/WHO, 2008).

Disease outbreaks associated with fresh produce consumption have become endemic and have been increasing as consumption of fresh produce increases (Murray *et al.*, 2018). In the United States of America (USA), outbreaks linked to consumption of fresh produce were reported to have increased from 14.8% to 22.8% from 1998 to 2007 (Wadamori *et al.*, 2017). Tomatoes contaminated with *Salmonella newport* were responsible for infections in 510 patients in 26 states of the USA in 2002. *Salmonella* has also been the cause of many other outbreaks associated with fresh produce (alfalfa sprouts, cucumber, papaya, cantaloupe and mangos) reported between 2006 and 2018, mostly in the United States (Jung *et al.*, 2014; Murray *et al.*, 2018). Recently, *Salmonella* outbreaks associated with consumption of pre-cut melon (137 cases reported of which 38 people were hospitalised) and papaya (81 cases reported of which 27 people were hospitalised) were reported in May and July 2019 respectively, in the United States (Centers for Disease Control and prevention (CDC, 2019a)). There has been reported *Listeria monocytogenes* outbreaks associated with frozen vegetables (nine cases reported, all nine hospitalised and three deaths were counted), cantaloupe (147 cases reported, reported, 143 hospitalised and 33 deaths were counted), beans sprouts (five illnesses reported, all five were hospitalised and two lost their lives) and caramel apples (35 cases reported, 34 were hospitalised and

seven deaths were counted) (CDC, 2019a). STEC has been more frequently associated with fresh produce compared to *Salmonella* and *L. monocytogenes*. A large *E. coli* O104:H4 outbreak was reported in 2011 in Germany in which over 4000 people were infected and about 850 had developed haemolytic uremic syndrome (HUS) and about 54 lives were lost (Beutin & Martin, 2012). This outbreak was associated with consumption of fenugreek sprouts (Beutin & Martin, 2012). STEC outbreaks continue to be a global concern, and many other STEC outbreaks associated with fresh produce have been reported (Jung *et al.*, 2014; Wadamori *et al.*, 2017; Murray *et al.*, 2018). A recent *E. coli* O157:H7outbreak linked with consumption of Romaine lettuce was reported between November and December 2019 in the United States, with 102 cases and 58 people hospitalised (CDC, 2019b).

In South Africa, there are no documented reports of food-borne outbreaks associated with fresh produce consumption. However, STEC and *Salmonella* have been isolated from fresh produce. *E. coli* O157:H7 has been found on carrots, spinach, onions and cucumbers collected from the Omathole District, Eastern Cape, South Africa (Abong *et al.*, 2008). *Salmonella* was detected on fresh produce (spinach and cabbage) sampled from informal and formal retail outlets In Johannesburg, South Africa (Du Plessis *et al.*, 2017). *Salmonella* was also isolated from contact surfaces within the fresh produce packinghouse Van Dyk et al., 2016). In this regard, fresh produce could be contaminated with *Salmonella* through contaminated contact surfaces. Nonetheless, there is limited information regarding the prevalence of pathogens on fresh produce from the Western Cape, South Africa.

Fresh produce can be contaminated while in the field, after harvesting, during transportation, processing and packaging as well as during food preparation by consumers (Brackett, 1999). Many factors such as contaminated soils, inadequately composted manure and insects can potentially contaminate fresh produce at production level (Rajwar *et al.*, 2016; Alegbeleye *et al.*, 2018). However, irrigation water with poor microbiological quality has been highlighted as one of the main factors contaminating fresh produce while in the field (Allende & Monaghan, 2015; Alegbeleye *et al.*, 2018). Some rivers in the Western Cape that are used to irrigate fresh produce were reported carrying high levels of microorganisms, and could potentially contaminate fresh produce (Britz *et al.*, 2012; Olivier, 2015). Contamination acquired before processing can prevail on the produce until it reaches the consumer's table. Therefore, some processing steps undertaken at the pack-house are meant to remove microorganisms, in order to supply consumers with fresh produce safe from microorganisms (Francis *et al.*, 2012; Zhou *et al.*, 2014). However, during processing, contaminated wash water, surfaces, packaging materials and workers hands can potentially contaminate the produce with enteric pathogens (Gil *et al.*, 2015; Rajwar *et al.*, 2016). Consequently, the produce might carry high levels of microorganisms by the time it reaches

the consumer's table. In a study done by Van Dyk et al. (2016) in Limpopo province, South Africa, tomato samples collected from the market were detected with higher microbial levels than those collected right from the field. The level of microorganisms were suspected to have increased during washing and packaging (Van Dyke et al., 2016). This highlights that fresh produce can get contaminated even during processing, and is a concern because most fresh produce is eaten raw without prior heat treatment that can inactivate microorganisms (Wadamori *et al.*, 2017). As a result, contaminated produce may transfer pathogens to the consumers. Pathogens such as STEC, *Salmonella* and *L. monocytogenes* are associated with serious morbidity and mortality (CDC, 2019a, Jung *et al.*, 2014; Wadamori *et al.*, 2017; Murray *et al.*, 2018). Although there are studies that have reported the prevalence of microorganisms on fresh produce, from farm to market, the potential contamination points along the production chain is not quite clear. Also, information regarding the microbiological quality of fresh produce sold in the Western Cape is still limited.

Another concern is the rising antimicrobial resistance within the *Enterobacteriaceae* family (Raphael *et al.*, 2011; Zurfluh *et al.*, 2015). It has been reported that *Enterobacteriacea*e can produce extended spectrum beta-lactamases (ESBLs) (Blaak *et al.*, 2014; Van Hoek *et al.*, 2015). The ESBLs cause resistance to many β-lactam antibiotics including the third generation cephalosporin (Ojer-Usoz *et al.*, 2013). The ESBL-producing *Enterobacteriaceae* were usually only associated with clinical settings, but they are now prevalent in many environments including the farming environments (Said *et al.*, 2015). In the farming environments, fresh produce can acquire the ESBL-producing organisms through contaminated soils, irrigation water and inadequately treated/composted animal manure (Said *et al.*, 2015; Van Hoek *et al.*, 2015). In a study done by Richter *et al.* (2019) in Gauteng province, South Africa, 79.2% of the produce isolates tested positive for ESBL-producing *Enterobacteriaceae*, and 75.3% of the isolates were confirmed carrying the β-lactamase genes. In addition, 96.1% of the tested produce isolated were detected with resistance to multiple antibiotics (Richter *et al.*, 2019). In a study done in the Western Cape, South Africa on fresh produce from informal markets by Laubscher (2019), some isolates were identified as ESBL producers. These results are worrisome and have highlighted the persistence of ESBL- producing organisms carrying the β-lactamase genes on fresh produce. These studies did not represent the produce sold in the formal retail sectors in the Western Cape, South Africa. Information regarding prevalence of ESBL-producing *Enterobacteriaceae* on fresh produce sold in the Western Cape formal retail sector is still limited.

The ESBL genes may then persist on fresh produce throughout the supply chain (Zurfluh *et al*., 2015). The fact that fresh produce is eaten raw could allow the ingestion of

organisms carrying ESBL genes which could colonise the humans gut and may exchange resistance genes to the bacteria found in the humans intestines (Van Hoek *et al.*, 2015). The ESBL genes cause resistance to a number of classes of antimicrobial drugs used to fight against bacterial infections (Blaak *et al.*, 2014). This can limit the use of available antimicrobial drugs and interfere with the treatment against bacterial infections (Pitout and Laupland, 2008).

The overall aim of this study was therefore, to determine the changes in the microbiological quality of fresh produce pre- and post-pack-house processing and at the formal point-of-sale, in order to identify potential contamination points along the supply chain. It was achieved through two objectives. The first objective was enumeration of microbial indicators (coliforms, *E. coli* and *Enterobacteriaceae*) from fresh produce sampled before and after pack-house processing steps, to determine the impact of processing on the microbial load of fresh produce. The second objective was enumeration of the microbial indicators as well as to testing for the presence of microbial pathogens: *Salmonella* and STEC and the ESBL-producing *Enterobacteriaceae* and the antimicrobial susceptibility on fresh produce samples collected pre- and post-pack-house processing steps as well as at the retail outlets.

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CHAPTER 2

LITERATURE REVIEW

BACKGROUND

Fresh produce is known with a significant role in nutrition and healthy diets (Fernanda *et al.*, 2013). In many countries, people have been encouraged to increase the intake of fruits and vegetables, in order to improve their health status (Food and Agriculture Organisation/World Health Organisation, 2008). This has then resulted in consumers demanding for more fresh produce, subsequently leading to increased fresh produce markets (Castro-Ibáñez *et al.*, 2017). In most African countries including South Africa, vegetables like cabbage, spinach, and tomatoes are eaten daily (Faber *et al.*, 2017). As the population grows, the demand for fresh produce also increases. Many people are becoming educated and aware of food safety; hence the microbial safety of fresh produce has become a concern worldwide (Van Boxstael *et al.*, 2013). South Africa is one of the African countries with a grown agricultural sector, and has been marketing fresh produce internationally for over a 100 years (Korsten *et al.*, 2015). However, the scarcity of potable water used for irrigation has implicated the water quality used for irrigation, posing contamination risks, therefore, this has raised concerns on the microbiological quality of fresh produce in South Africa (Du Plessis & Korsten, 2015). Nonetheless, it is not only irrigation water that contributes to poor microbial quality of fresh produce, but the unhygienic processing and handling of fresh produce along the supply chain also plays a role in contaminating fresh produce with food-borne pathogens (Nyenje *et al.*, 2012; Bartz *et al.*, 2017).

Retained flavours and nutrients are important parameters considered during food preparation (Qadri *et al.*, 2015). This has therefore led to consumers preparing their vegetables with less heat or no heat treatment (Thunberg *et al.*, 2002). However, pathogens may survive and get transmitted to human through ingestion of vegetables and fruits, hence, posing health risks to consumers, since there is no effectual pathogen elimination treatment involved (Ramos *et al.*, 2013). Vegetables like lettuce, spinach, cabbage, broccoli and other vegetables used for salads are eaten raw or minimally processed with no further treatment, hence implicated with food-borne diseases (Sujeet & Vipin, 2015). In many studies *Salmonella, Escherichia coli (E. coli), Staphylococcus aureus, shigella spp. and L. monocytogenes* have been the most isolated from ready to eat fresh produce salads (Seow *et al.*, 2012; Mir *et al.*, 2018), and they have also been implicated in most the reported outbreaks associated with fresh produce (Suslow *et al.*, 2003; Alegbeleye *et al.*, 2018).

FRESH PRODUCE BENEFITS TO HUMANS

Fresh produce is very important in terms of food supply and consumers' wellbeing. Balancing diets with fresh produce (fresh fruits and vegetables) provides consumers with vitamins, minerals, fibre, essential micronutrients, proteins and phytochemicals (Miller *et al.*, 2017; Septembre-malaterre *et al.*, 2018). These nutrients and phytochemicals promote good health by inhibiting the occurrence of obesity, cardiovascular diseases, diabetes, cancer, respiratory diseases, as well as vitamins and micronutrients' deficiencies which are responsible for some health issues (FAO, 2015). According to (Boeing *et al.*, 2012) it was proved that fresh vegetables and fruits consumption leads to reduced hypertension, chronic heart diseases, and stroke. Fresh produce consumption is also associated with weight loss hence reducing obesity (Schroder, 2010). Studies have also indicated that, a regular diet containing fruits can lower eye problems, osteoporosis and lung diseases (Boeing *et al.*, 2012). Therefore, it is highly recommended for people in both developed and developing countries to keep consuming fresh produce in the right amount, to improve their health status. The World Health Organisation (WHO) has recommended an intake of 400g per day (Schreinemachers *et al.*, 2018).

Fresh produce is not only important for human food, nutritional and health benefits, but it also contributes to the country's economy. The expansion of fresh produce production in different countries leads to job opportunities, increased fresh produce markets, expanded international trading; thereby contributing to the national economy (Schreinemachers *et al.*, 2018). South Africa exports its agricultural products to about 92 countries, and 50% of these products are fresh fruit (Fresh fruit directory, 2018). In 2018, an extra $2.5 billion was generated from fresh produce (citrus fruit and other fruits, as well as vegetables) exported from South Africa (Fresh fruit directory 2018).

FOOD-BORNE OUTBREAKS ASSOCIATED WITH FRESH PRODUCE

Due to the growing awareness about nutritional and health benefits of fresh produce, there has been an increase in consumption of fresh produce worldwide (Jung *et al.*, 2014). However, eating fresh produce contaminated with pathogens is implicated with food-borne illnesses (Mir *et al.*, 2018). With trending healthy lifestyles, many people opt to eat raw fresh produce salads (Sujeet & Vipin, 2015). However, this could put consumers' health at risk, because pathogens may persist on produce, and can be carried on to the consumers because there is no effective treatment method involved, (Mercanoglu Taban & Halkman, 2011; Castro-Ibáñez *et al.*, 2017; Kase *et al.*, 2017).

Food-borne outbreaks linked with contaminated fresh produce consumption have been reported globally, and reports have showed a growth in outbreak number (Jung *et al.*, 2014; Franz *et al.*, 2018). Over the past years, food-borne illness outbreaks were mainly caused by food products other than fresh produce, such as meat, seafood and dairy products (Korir *et al.*, 2016). However, due to the increased consumption of raw or minimally processed contaminated fresh produce (Castro-Ibáñez *et al.*, 2017), fresh produce is becoming a frequent cause of food-borne illness outbreaks (Murray *et al.*, 2018). Outbreaks associated with products such as carrots, tomatoes, spinach, lettuce, cabbage, radish, broccoli, cucumber, and other leafy vegetables and fruits have been reported globally (Van Boxstael *et al.*, 2013; Sujeet and Vipin, 2015; Mir *et al.*, 2017).

Data collected from the United States of America between 1998 and 2005, have indicated leafy vegetables and herbs as a leading produce, accounting for 70% of the fresh produce outbreaks reported within that period (FAO/WHO, 2008) In Brazil, during the same period, 75% of overall fresh produce outbreaks were linked with leafy vegetables and herbs (EFSA, 2014). About 502 outbreaks associated with green leafy vegetables salads reported between 1973 and 2006, have caused 18 242 illnesses and 15 deaths (FAO/WHO, 2008). Furthermore, 68 food-borne outbreaks have occurred in America between 2006 and 2014, of which 16 of them were attributed to fresh produce, and 38% of these fresh produce outbreaks were linked to sprouts (Jung *et al.*, 2014). A huge outbreak associated with fenugreek sprouts caused by *E. coli* O104:H4 was reported in Germany in 2011 responsible for over 4 000 illnesses, more than 850 haemolytic uremic syndrome cases, and loss of 54 lives (Beutin and Martin, 2012; Jung *et al.*, 2014). There are quite a number of microbial pathogens associated with food-borne diseases, but the most associated with fresh produce, are *Escherichia coli* (0157:H7), *Salmonella* spp, *Shigella* and *Listeria monocytogenes* (Mercanoglu Taban and Halkman, 2011; Sujeet and Vipin, 2015; Castro-Ibáñez *et al.*, 2017; Mir *et al.*, 2018).

These outbreaks impact consumer' trusts of several products negatively; it is therefore good to engage in preventative measures, which could lower the risk of fresh produce contamination to ensure food safety in both developed and developing countries, consequently enhancing good international trade between countries (Wadamori *et al.*, 2017; Murray *et al.*, 2018).

Table 2.1 Produce and pathogens involved in food-borne illnesses outbreaks reported in the United States and the European Union (2012 to 2017) *(Jung et al., 2014; Wadamori et al., 2017; Murray et al*., 2018)

Year	Produce involved	Pathogens	No. of cases
2017	Papayas	*Salmonella* Kiambu, Thompson, Agona	173
2016	Rock melon	*Salmonella* Hvittingfoss	97
	Pre-packaged lettuce	*Salmonella* anatum	144
	Imported salads	*E. coli* O157	161
	Packaged salads	*L. monocytogenes*	19
	Frozen vegetables	*L. monocytogenes*	9
	Frozen strawberries	Hepatitis A	143
2015	Tomato	*Salmonella* Newport	115
	Cucumber	*Salmonella* Poona	907
	Imported frozen strawberries	Hepatitis A	19
	Imported cucumber	*Salmonella* Poona	>900
2014	Prepackaged caramel apples	*L. monocytogenes*	32
	Fresh vegetables	Yersinia pseudotuberculosis	334
	Mung beans sprouts	*L. monocytogenes*	5
	Lettuce, cucumber	Enteroinvansive *E. coli* O96	50
	Salads	*Salmonella Singapore*	4
	Raw clover sprouts	*E. coli* O121	19
	Coriander	*Cyclospora cayetanesis*	304
	Caramel Apples	*L. monocytogenes*	35
	Cucumbers	*Salmonella enterica* Newport	275
2013	Shredded lettuce	*E. coli* O157:H7	30
	Imported cucumber	*E. coli* O157:H7	33
	Salad mix	*Cyclospora cayetanensis*	631
	Imported pomegrated seeds	Hepatitis A Virus	165
	Imported cucumber	*Samonella*	84
	Bean sprouts	*S*. enteritidis	87
2012	Mango	*Salmonella enterica* Braenerup	157
	Cantaloupe	*Salmonella enterica* Typhimurium and Newport	261
	Romaine Lettuce	*E. coli* O157:H7	24
	Organic spinach/spring mix blend,	*E. coli* O157:H7	33
	Cucumbers	*Salmonella enterica* Sainrpaul	84

PATHOGENIC MICROORGANISMS ASSOCIATED WITH FRESH PRODUCE

Background

Food-borne illnesses are caused by the predominance of pathogenic microorganisms on consumers' food. Most of the pathogenic microorganisms can be destroyed by heat (Fox *et*

al., 2018). However, it is quite challenging when it comes to fresh produce, because most fresh produce is eaten raw or processed with less heat treatment, which is insufficient to kill the microorganisms (Olaimat and Holley, 2012). It is for these reasons, a steady growth in food-borne illnesses attributed to fresh produce has been observed (Johnston *et al.*, 2006; Franz *et al.*, 2018).

There is quite a range of pathogenic microorganisms implicated in food-borne illness outbreaks, and testing for the presence of each and every one of them in food is difficult, expensive, time-consuming, and pathogens may be present in low number or could be absent (Ssemanda *et al.*, 2017). Therefore, microbial indicators are used to determine the microbiological quality of food/fresh produce, and also used to give an indication of type of organisms present in the food/produce (Eden, 2014). Studies and reports on food-borne illnesses linked with fresh produce have indicated bacterial pathogens, viruses and parasites as causative agents of the reported food-borne diseases worldwide (Sivapalasingam *et al.*, 2004; FAO/WHO, 2008; Olaimat and Holley, 2012; Van Boxstael *et al.*, 2013; Ssemanda *et al.*, 2017).

Indicator organisms

These are organisms used to reveal the hygienic conditions of food/fresh produce, or the processing environment (Eden, 2014; Badalyan *et al.*, 2018). The use of indicator organisms employs assessing the numerical level at which the organism is present in food, against the limit guidelines set for a specific food (Halkman & Halkman, 2014). It is widely reported that fresh produce can get contaminated at any point along the supply chain from farm to consumer (Faour-Klingbeil *et al.*, 2016; Alegbeleye *et al.*, 2018), due to inadequate sanitation practices along the supply chain (Halkman & Halkman, 2014). Therefore, indicator organisms like total coliforms, *E. coli, Enterobacteriaceae,* non-*monocytogenes Listeria,* total yeast and mould, and total viable cell count, are used in food industries, as markers of faecal contamination, processing failure, inadequate heat processing, and general sanitary levels (Eden, 2014; Halkman & Halkman, 2014; Ssemanda *et al.*, 2017).

Enterobacteriaceae

Enterobacteriaceae is a group of pathogenic and nonpathogenic gram-negative bacteria, found in human and animals' intestinal tract, soils, vegetable matters, and in marine environments (New South Wales (NSW) Food Authority, 2009). *Enterobacteriacreae* are further classified as rode-shaped, with the ability of growing in both aerobic and anaerobic conditions, glucose and other sugars fermenters, convert nitrates to nitrites, non-oxidase producing bacteria but they produce catalase exclude Plesiomonas, and they do not form

spores (Osaili *et al.*, 2018). *Enterobacteriaceae* include the entire coliform and *E.coli* group, as well and the gram-negative food-borne pathogens such as *Salmonella* spp., *Shigella,* and *Yersinia enterocolitica* (Kaushik *et al.*, 2018).

Total coliforms were primarily used as an indicator for microbial hygeine in the processing industries. Studies have however found the use of coliforms in processed products inadequate to represent a number of gram-negative bacteria (Hervert *et al.*, 2016). Therefore, *Enterobacteriaceae* was suggested as an alternative indicator, following the advantage that, its detection is more inclusive of the total coliforms, and other bacteria of the *Enterobacteriaceae* family, which are non-coliforms (Wiedmann *et al.*, 2016). *Enterobacteriaceae* in food industries is used as an indicator to reflect sanitary levels, post-processing contaminations or inadequate heat processing (Eden, 2014; Ojer-Usoz *et al.*, 2013; NSW Food Authority, 2009). The presence of *Enterobacteriaceae* in food at levels higher than the set guidelines gives an indication of poor sanitation, or underprocessing of food (Eden, 2014). The test methods for *Enterobacteriaceae* detection are carried out by enumaration, using violet red bile glucose (VRBG) agar containing inhibitory components (bile salts and glucose) which suppress the growth of unwanted organisms (Halkman and Halkman, 2014; Ssemanda *et al.*, 2017).

Enterobacteriaceae can prevail on fresh produce in large numbers due to the fact that they exist in a wide range of environments. They have been detected on fresh produce sampled from the field, at market, upon arrival at food service establishments, and after salad preparations at 5.8, 6.3, 6.0, and 3.3 log $CFU.g^{-1}$ respectively (Ssemanda *et al.*, 2017). Nguz *et al.*, (2005) have also detected *Enterobacteriaceae* on shredded iceberg lettuce in a range of 1.6 $\log_{10}$ - 9.8 $\log_{10}$ $CFU.g^{-1}$.

Total coliforms

The use of coliforms started back in 1914, as an indicator of microbiological quality and safety of water used for drinking (Wiedmann *et al.*, 2016). Coliform bacteria is frequently used in food and water as an indicator of product quality. They are described as “facultative anaerobic, gram-nagative, non-spore forming rod-shaped bacteria”, with the ability of fermenting lactose producing gas and acid at 35^{o}C within 48 h (Eden, 2014; Hervert *et al.*, 2016). They contain an enzyme called *β-galactosidase* which breaks lactose into glucose and galactose, and they are non-oxidase producing bacteria (Adam & Mæhlum, 2012). Coliforms constitute four members namely *Citrobacter*, *Enterobacte*r, *Escherichia* and *Klebsiella*, found natually in human and animals intestines, as well in soil and water (Colclasure *et al.*, 2015). The use of coliforms was introduced over a 100 years ago, to test faecal contaminations in water (Leclerc *et al.*, 2001). This was then later adapted by many

food industries to reveal faecal contaminations, and poor sanitary conditions in the food processing facilities (Trmčić *et al.*, 2016). However, the fact that coliforms are found in a wide range of environmment, their presence does not always indicate faecal contamination (Leclerc *et al.*, 2001; Damyanova *et al.*, 2016). Therefore, coliforms can only be used as indicator and not as index organisms (Trmčić *et al.*, 2016). By definition, indicator organisms are those whose presence indicates poor processing sanitary conditions, whereas index organisms are those whose presence gives an indication of the possibility of an ecologically similar pathogen to occur (Leclerc *et al.*, 2001; Damyanova *et al.*, 2016; Trmčić *et al.*, 2016).

Coliforms can be grouped into three categories based on their origin, to allow the correct interpretation of coliforms' test results (Leclerc *et al.*, 2001; Trmčić *et al.*, 2016). These groups are (a) Psychrotolerant environmental coliforms, originated from contaminated waters, and mainly found on vegetables sources, (b) thermotolerant faecal coliforms, originated from faecal matters, and (c) ubiquitous coliforms including thermotolerant coliforms, found in human and other warm blooded animals' intestines, as well as natural environment (Leclerc *et al.*, 2001). Although the presence of coliforms does not indicate the presence of similar ecological pathogen, enteric pathogens are likely to occur where coliforms exist in a large number (Lues & Van Tonder, 2007).

Previous guideline limits set by the South African Department of Health (DoH, 2002) (currently under review) suggest not more than 200 $CFU.g^{-1}$ coliforms in ready-to-eat fresh fruits and vegetables. However, high concentrations of coliforms have been detected in both water used for irrigation, and on fresh produce (Thunberg *et al.*, 2002; Roth *et al.*, 2018). Studies done in South African river water used for irrigation have found coliforms at unacceptable levels exceeding the South African Department of Water Affairs (DWA) criteria for safe irrigation water (<1000 $cfu.100\ mL^{-1}$) (Gemmell & Schmidt, 2012). These coliforms could be transfered to the fresh produce through irrigation, and may grow on fresh produce when exposed to temperatures that favour their growth. In a study done by Van Dyk *et al*. (2016) in South Afica, coliforms were not observed on tomatoes sampled from two farms at four weeks prior to harvest, however, counts were detected at two weeks prior to harvesting, in low levels (1.0 to 2.0 log $CFU.g^{-1}$), which had then increased from the washing step (2.2 log $CFU.g^{-1}$ on tomatoes from Farm 2), and the highest levels were observed at the market (ranged from 1.9 to 6.2 log $CFU.g^{-1}$) (Van Dyk et al., 2016). In a study done by Nguz *et al.* (2005) on microbiological quality of fresh-cut vegetables organically produced in Zambia, coliform counts were observed ranging between 2.2 $\log_{10}$ $CFU.g^{-1}$ - 5.9 $\log_{10}$ $CFU.g^{-1}$. Both studies have indicated coliform levels exceeding the previous South African Department of Health (DoH, 2002) guidelines (under review) for coliform on fresh fruits and vegetables intended to be consumed raw.

Faecal coliforms

Faecal coliforms are a subgroup of the total coliform group, that have all coliforms' features, but they produce lactose at high temperatures ranging from 44.5 ^{o}C to 45.5 ^{o}C at 24-48h, and they do not live freely outside the hosts for a long time like coliforms (Eden, 2014). Faecal coliforms are microflora of the intestinal tract of humans and animals, hence specifically used to reflect faecal contaminations (Apte *et al.*, 1995; Castro-Rosas *et al.*, 2012; Eden, 2014). Faecal coliforms have been used in water, fresh produce, dairy products, and other food materials to indicate faecal contaminations (Britz *et al.*, 2013; Eden, 2014; EFSA, 2014).

Studies conducted on Western Cape rivers (Plankernburg and Eerste) used for irrigation, have found these rivers faecally contaminated at high levels exceeding the guideline limits (1000 cfu.100 mL^{-1}) set by the South African Department of Water Affairs (DWA), and by the World Health Organization (WHO) (Britz *et al.*, 2013). These rivers are used for irrigating crops and could carry contaminations to the crops. In a study conducted by Gemmell and Schmidt, (2012) in South African river water used for fresh produce irrigation in Sobantu, faecal coliforms found in water, as well as on fresh produce were up to 1.6×10^{6} CFU.100 mL^{-1} and 1.6×10^{5} CFU.g^{-1} respectively.

Faecal coliforms consist both pathogenic bacteria and non-pathogenic bacteria. *Escherichia coli* and faecal *Enterococci* are examples of faecal indicators commonly used in water and food (Horan, 2003; Gemmell & Schmidt, 2012; Halkman & Halkman, 2014). For an organism to be used as an indicator of faecal pollution, it should meet the following criteria: it should be an organism of the interstinal tract, exist in faeces in a large amount for easy detection after dillution, should be able to stay alive in the test sample, and be detectable even when it is present in low levels (Halkman & Halkman, 2014).

Escherichia coli

Escherichia coli (E. coli) is Gram-negative bacteria (Alharbi *et al.*, 2018), which was discovered by Theodore Escherich, and given the preference of being a biological indicator for water safety, in the 1980s (Leclerc *et al.*, 2001). It is found in the gastrointestinal tract of warm-blooded animals, as well as of humans (Adam & Mæhlum, 2012; Kolm *et al.*, 2018). This organism has all coliforms and faecal coliform features, however, the absence of urease and the presence of *B-glucuronidase* has differentiated it from faecal coliforms (Eden, 2014). *E.coli* appears in mammal faeces in a large number of 10^{9} per gram, and can be transferred to the environment through faeces, consequently contaminating surface waters, soils, and crops (Horan, 2003; Adam & Mæhlum, 2012).

E. coli has been widely acknowledged as a good indicator of faecal contamination in water (Leclerc *et al.*, 2001). However, given that preference, its application as an indicator has been expanded to many other food products such as milk, meat and vegetables, as a faecal contamination indicator as well as an index organism indicating the potential presence of a pathogenic *E. coli* (Beerens *et al.*, 2000; Patel *et al.*, 2014; Korir *et al.*, 2016; Jaja, 2018; Kundu *et al.*, 2018). The presence *of E. coli* at levels exceeding the guidelines for safe food, can be health threatening, as it can be associated with the presence of similar ecologically enteric pathogen. In South Africa, the concentration of *E.coli* on fresh fruits and vegetables to be eaten raw is expected to be 0 $CFU.g^{-1}$ (DoH, 2002). Contaminated water used for irrigation can be a transmission vehicle of *E. coli* on fresh produce (Pachepsky *et al.*, 2011; Jongman & Korsten, 2017). Studies done in South Africa concerning the concentration of *E.coli* in river water used for irrigation and on fresh produce have found high levels of *E. coli* exceeding the guideline limits, in rivers water, and on some vegetables irrigated with contaminated water (Britz *et al.*, 2012; Korsten *et al.*, 2015). In another study done by Du Plessis *et al.*, (2015), *E.coli* was found in river water at concentrations ranging between 1.59 log $CFU.100\ mL^{-1}$ and 4.66 log $CFU.100\ mL^{-1}$. Beharielal *et al.* (2018), have also reported poor microbial quality of irrigation water used on fresh produce by a number of South African farmers, and have also found *E. coli* on fresh produce (lettuce, parsley, carrots, and spinach) at levels ranging between 2.2 to 49 $MPN.g^{-1}$. Faecal contamination (*E.coli*) of irrigation water and fresh produce is not only a burden in South Africa but it is a wolrdwide issue. This has been reported by many studies done in Africa (Nguz *et al.*, 2005; Shenge *et al.*, 2015), as well as outside Africa (Thunberg *et al.*, 2002; Johannessen *et al.*, 2015; Allende *et al.*, 2018; Kundu *et al.*, 2018).

Bacterial pathogens

Escherichia coli (E. coli)

Although most of the *E. coli* strains are normal microflora of a healthy interstinal tract (Farrokh *et al.*, 2013; Baker *et al.*, 2016), some strains have the ability of acquiring virulence factors, which are causative agents of the gastrointestinal or extraintestinal diseases (Farrokh *et al.*, 2013; Müller *et al.*, 2016). The pathogenic *E. coli* are divided into six pathotypes depending on their virulence type (Castro-Rosas *et al.*, 2012). These pathotypes include enteropathogenic (EPEC), enterotoxigenic (ETEC), enterohaemorrhagic (EHEC) also called Shiga toxin (stx) producing *E. coli* (STEC), enteroaggregative (EAEC), diffusely adherent (DAEC), and enteroinvasive (EIEC) *Eschericia coli* (Aijuka *et al.*, 2018).

In early 1980s, Shiga toxin-producing *E. coli* (STEC) strains emerged as major life threatening food-borne pathogens, associated with diarrhea in humans which eventually develop into severe gastrointestinal and systemic diseases known as hemorrhagic colitis (HC), and hemolytic uremic syndrome (HUS) (Jinneman *et al.*, 2012; Baker *et al.*, 2016). STEC produces one or more virulence genes (*stx*1 and *stx*2) and other variants (Berger *et al.*, 2010; Fox *et al.*, 2018). STEC also contains a chromosomal gene *eae* which codes for intimin (Baker *et al.*, 2016).

There have been a number of food-borne outbreaks associated with STEC infections, linked to ingestion of undercooked food, raw milk and raw fresh fruits and vegetables (Jinneman *et al.*, 2012; Jung *et al.*, 2014; Murray *et al.*, 2018). Globally, STEC has been isolated from leafy vegetables (Khalil *et al.*, 2015), human, animals, food and the environmment (Li *et al.*, 2016; Kanengoni *et al.*, 2017; Dias *et al.*, 2019). Six STEC serotypes (O26, O103, O146, O128, O145 and O157) have been identified, frequently associated with infections in humans (Fox *et al.*, 2018; Dias *et al.*, 2019). Howerver, *E. coli* O157:H7 has been recognised as a predominant serotype associated with many reported outbreaks and sporadic cases of hemorrhagic colitis and hemolytic-uremic syndrome (Bryan *et al.*, 2015). Nonetheless, the first large STEC outbreak was caused by *E. coli* O104:H4, which was reported in Germany in 2011, with 4 075 cases, and about 50 deaths (Jinneman *et al.*, 2012; Jung *et al.*, 2014).

Shiga toxin-producing *E.coli* outbreaks are frequently reported in America. In 2018, an *E. coli* O157:H7 outbreak associated with consumption of Romaine lettuce was reported in Canada, with 62 reported cases from 16 States and 25 people were hospitalized (CDC, 2018). Another *E. coli* O157:H7 oubreak was reported in the United States with 210 cases, 96 people being hospitalised of which 27 people developed hemolytic uremic syndrome (HUS), and five deaths were counted (CDC, 2018). This outbreak was also linked with Romain lettuce. Again in 2018, there were 18 cases of STEC (*E. coli* 026) infection reported in the United states, which were associated with ground beef. As a result, four people were hospitalised, one person has developed HUS, and one death was counted (CDC, 2018). An STEC outbreak caused by *E. coli* O103 strain was recently reported in the United States (CDC, 2019).

In South Africa, STEC (*E. coli*) O157: H7 has been isolated from beef, pork, water and human patients (Galane & Le Roux, 2001; Ateba and Mbewe, 2011), as well as on faeces of dairy cattle (Iweriebor *et al.*, 2015). STEC has also been isolated from fresh produce in South Africa. In a study done by Abong *et al.* (2008), on the prevalence of *Escherichia coli* O157:H7 in vegetables sampled from the Amathole District in the Eastern Cape Province of South Africa, presumptive *E. coli* O157 counts were observed in carrots (9

× 10^3 CFU.g^{-1}), spinach (from 4.1 × 10^4 to 1.6 × 10^6 CFU.g^{-1}), onions (1.6 × 10^5 CFU.g^{-1}), as well as on cucumber (1.3 × 10^3 CFU.g^{-1}); and four of the total (39) vegetable samples analysed were found positive with *E. coli* O157:H7.

Animals have been identified as STEC reservoirs, largely prevailing in domestic ruminant animals like cattle (Khalil *et al.*, 2015; Dias *et al.*, 2019). Therefore, environments, waters used for irrigations, and agricultural soils may acquire STEC from animal faeces, consequently transferring the pathogen to the fresh produce (Khalil *et al.*, 2015; Dias *et al.*, 2019). Fresh produce can however, get contaminated with STEC directly through faecal contaminations (Farrokh *et al.*, 2013). As a results, fresh produce that is eaten raw, or subjected to minimal processing, may expose STEC to humans, resulting into diseases normally at a low infection dose of 5-50 cells (Farrokh *et al.*, 2013). Shiga toxin producing *E. coli* infections may result bloody diarrhea, severe hemorrhagic colitis, and hemolytic-uremic syndrome (Ackers *et al.*, 1998; Harris *et al.*, 2003; Hussein & Bollinger, 2005). However, the symptoms might differ depending on the *E. coli* strain involved (Farrokh *et al.*, 2013).

Salmonella

Salmonella is a gram-negative facultative anaerobic bacteria consisting of two species: *Salmonella enterica* serovars and *Salmonella bongori* (Lamas *et al.*, 2018; Persad & LeJeune, 2018). The main concern has been *Salmonella enterica* which is pathogenic, causing Salmonellosis in humans (Lamas *et al.*, 2018). *Salmonella enterica* is divided into six subspecies, *Salmonella enterica (S. enterica*) *Salmonella salamae*, *Salmonella arizonae*, *Salmonella diarizonae*, *Salmonella houtenae,* and *Salmonella indica* (Lamas *et al.*, 2018; Persad and LeJeune, 2018a). Subspecies *S. enterica* is further classified with over 1 500 serovars (Fox *et al.*, 2018; Lamas *et al.*, 2018), of which serovars *enteritidis* (*S. enteritidis*), *typhimurium* (*S. typhimurium*), Newport and Stanley, have been commonly associated with many cases of food-borne infections in humans (Esmailnejad *et al.*, 2019). *Salmonella enterica* subspecie *S. enterica* is a microflora of warm blooded animals. Whereas, other subspecies are mostly found in cold-blooded animals, and are hardly associated with human illnesses (Salmonellosis) (Lamas *et al.*, 2018).

Salmonella contaminates environments through animal droppings, and may persist in the environment for many months, even over 12 months (Persad & LeJeune, 2018). As a result, fresh produce can potentially acquire *Salmonella* while in the field or throughout the supply chain, consequently, posing health risks to consumers (Harris *et al.*, 2003; Mir *et al.*, 2018). Infections by *Salmonella* are symptomised by vomiting, abdominal cramps, diarrhea, headaches and fever; which may appear from six to 48 hr after eating contaminated food and may persist for four to seven days (Harris *et al.*, 2003; Monaghan, 2010; Mir *et al.*,

2018). *Salmonella* has been one of the leading pathogens causing gastrointestinal illness in humans worldwide (Fox *et al.*, 2018). Varieties of food such as dairy products, meat and meat products, fish, fruits and vegetables and poultry products have been reported to be implicated with Salmonellosis outbreaks worlwide, (EFSA, 2017). According to Beuchat (1996), cases of *Salmonella* were mostly associated with meat and poultry products than fresh produce. However, studies have indicated an increase in salmonellosis cases resulting from consumption of contaminated fresh produce (Harris *et al.*, 2003; Monaghan, 2010). Several *Salmonella* outbreaks associated with sliced tomatoes, sprouts, sliced watermelon, sliced cantaloupe, cucumber, mango, papayas, and unpasteurised orange juice have been reported (Brackett, 1999; Awang Salleh *et al.*, 2003; Jung *et al.*, 2014; Murray *et al.*, 2018). In Brazil, 47% of the food-borne disease outbreaks reported were caused by *Salmonella* (Sant *et al.*, 2014). In 2015, *Salmonella* was one of the leading cause of food-borne infections in humans in the European Union, accounting for 94 625 confirmed cases and 126 reported deaths (Lamas *et al.*, 2018).

Cases of *Salmonella* infections have also been reported in South Africa. In 2006, an oubreak of *Salmonella* infection among school teachers, associated with consumption of food prepared by the school kitchen was reported in Mpumalanga; known to have been caused by *S. enterica* serotype *Salmonella* Virchow (Smith *et al.*, 2007). Seven other food-borne illness outbreaks caused by *Salmonella enteritidis* were reported in South Africa from 2013 to 2015, from six provinces (Guateng, limpopo, Eastern Cape, Mpumalanga, Free state, and KwaZuluNatal) (Muvhali *et al.*, 2017), shown in the Table 2.2.

Table 2. 2 Seven *Salmonella Enteritidis* outbreaks reported in South Africa from 2013 to 2014 (Muvhali et al., 2017).

Outbreak	Outbreak year	Province	No. of cases	No. of patients hosiptalized	No. of death	Products
1	2013	KZN	2	0	0	Goat meat
2	2013	MP	unknown	Unknown	unknown	Unknown
3	2014	LP	65	8	0	Unknown
4	2014	MP	46	6	0	Unknown
5	2014	FS	80	6	Unknown	Unknown
6	2014	EC	unknown	Unknown	Unknown	Unknown
7	2015	GA	4	4	0	Unknown

No=number; Gauteng (GA), Limpopo (LP), Mpumalanga (MP), Eastern Cape (EC), Free State (FS), KwaZulu-Natal (KZN)

Salmonella has also been isolated from fresh produce cultivated in South Africa. In a study by Du Plessis *et al.* (2017), on the microbiological quality of spinach and cabbage

purchased from the informal and formal retail trade in Johannesburg, South Africa, *Salmonella* spp was detected in 5% of the 180 tested samples. Another study that was conducted in South Africa by Van Dyk (2016), on "Microbiological food safety status of commercially produced tomatoes from production to market" analysed *Salmonella* Typhimurium from tomatoes, and found all tomatoes and soil samples free from *Salmonella* Typhimurium. However, *Salmonella* was detected on contact surfaces in the packinghouse, and this could be transferred to the produce during processing. In a study done by De Bruin *et al.* (2016), the microbiological quality of fresh basil was assessed from the production point to the retail point of sale in Gauteng and Northwest Provinces of South Africa; this study found four samples collected at the processing facilities positive with *Salmonella Typhimurium*, of which three of them were collected from basil ready for the packing line, and one from basil to be dispatched. This is proving that fresh produce indeed can get contaminated along the supply chain. Nguz *et al.* (2005), has evaluated the microbiological quality of fresh-cut vegetables (mixed vegetables and green beans) organically produced in Zambia, and *Salmonella* spp. was detected in 23.1% of the samples analysed. From these studies, it is clear that fresh produce can acquire contamination during processing, as a result, microorganisms may persist and proliferate on the produce, and those produce eaten raw may transmit the organisms to humans.

Shigella

Shigella is a pathogenic bacterium which causes an infectious disease called Shigellosis. *Shigella* belongs to the *Enterobacteriaceae* family, and is further classified as a facultative anaerobic, non-motile, and rod shaped Gram-negative bacterium (Warren *et al.*, 2006; Lee & Kang, 2016). *Shigella* comprises of four serogroups: *Shigella dysenteriae* (serogroup A), *Shigella flexneri* (serogroup B), *Shigella boydii* (serogroup C) and *Shigella sonnei* (serogroup D) (Warren *et al.*, 2006, 2007; Lee & Kang, 2016). These serogroups are all regarded as pathogenic, but they do not have the same epidemiology (Lee & Kang, 2016). *Shigella* is a natural habitant of the gastrointestinal tract of humans, hence can only be transmitted by faecal contamination. This bacteria is commonly transmitted from person to person, due to its very low infective dose, which ranges from 10^1 to 10^4 cells (Warren *et al.*, 2006; Chen, 2018). Contaminated persons and insects like flies can transmit *Shigella* to food and water when they get in contact (Chen, 2018). The presence of *Shigella* on fresh produce eaten raw can be threatening, because Shigellosis can occur from a very low infectious dose (Cohen *et al.*, 2019). In addition, when contaminated food is eaten, *Shigella* has the mechanisms of surviving the acids found in the stomach, as well as the competitive microbiota in the intestines, thereby able to move through the interstinal tract until the colon, where it invades

the epithelium of the large intestines (Warren *et al.*, 2006; Chen, 2018). However, the invasion and encoding of *Shigella*'s virulence plasmid polypeptides occurs at 37^{o}C (Warren *et al.*, 2006). Shigellosis infections are manifested by bloody diarrhea, abdominal pain, fever, and malaise which might be exprienced within a short length of 1 to 2 days after ingestion of contaminated food. People with a compromised immune system, old aged people, children, and pregnant women are more susceptible to Shigellosis (Warren *et al.*, 2007; Lee & Kang, 2016).

Food-borne outbreaks attributed to shigellosis have been reported globally. In the United States, shigellosis was reported as the third most popular food-borne bacterial infection; and close to 14 000 cases of Shigellosis are reported yearly (Lee & Kang, 2016). In 2016, *Shigella* was recorded with 212 438 deaths from all ages, and it was recognised as the second leading cause diarrhea deaths (Khalil *et al.*, 2018). Shigellosis outbreaks linked with food processed under low heat treatment or prepared by hands, and those served raw have been reported (Warren *et al.*, 2006). It has also been isolated from fresh produce such as green onion, lettuce, parsely and salad vegetables (Brackett, 1999).

Listeria monocytogenes

Listeria monocytogenes (L. monocytogenes) is a *Listeria* species responsible for listeriosis infections in humans (Monaghan, 2010; Fox *et al.*, 2018; Kljujev *et al.*, 2018). *Listeria monocytogenes* is a Gram-positive non-spore-forming facultative anaerobe bacteria (Ferreira *et al.*, 2014), initially identified in 1927 when it affected guinea pigs and rabbits (Radoshevich & Cossart, 2018). *Listeria monocytogenes* was only recognised as a food-borne pathogen in the 1980s (Radoshevich and Cossart, 2018).This organism is widely disseminated in the environment, it is found in the gut of humans and other warm blooded animals, in soils, water and fresh fresh produce (Kljujev *et al.*, 2018). *Listeria monocytogenes* can grow at very low temperatures, and can survive low pH, as well as high salt concentrations (Ferreira *et al.*, 2014; Radoshevich & Cossart, 2018). This enables *L. monocytogenes* to survive treatments fresh produce is exposed to during processing, subsquently standing a great chance of persisting and proliferating on fresh produce (Ferreira *et al.*, 2014). As a result, it becomes very difficult to eliminate *L. monocytogenes* from the environment, especially in the food processing environments, (Buchanan *et al.*, 2017; Kljujev *et al.*, 2018; Neha *et al.*, 2018).

The infective dose of *L. monocytogenes* ranges from 10^2 to 10^9 cells (Kljujev *et al.*, 2018). Consumption of food contaminated with *L. monocytogenes* may lead to mild or severe gastroenteritis, or bacterial sepsis (especially in individuals that are immunocompromised, old aged group, children and pregnant women), which may then

result into meningitis, fetus infection, subsquently resulting into pregnancy complications or miscarriage (Radoshevich & Cossart, 2018).

Food-borne illness caused by *L. monocytogenes* have been reported in both developed and developing countries. According to the EFSA (2017), there was an increase in Listeriosis cases reported between 2008 and 2016. About 2 161 Listeriosis cases were reported in 2014 (EFSA, 2014). In 2016 about 2 536 Listeriosis were reported in the *United states* (EFSA, 2017). These cases were associated with food products like smoked fish, soft and semi-soft cheeses, ready-to-eat meat and hard cheeses (Buchanan *et al.*, 2017; EFSA, 2017). However, listeriosis oubreaks associated with pre-cut celery, ice cream, cantaloupe, mung bean sprouts, stone fruits, caramel apples, were reported in the United States between 2010 and 2014 (Buchanan *et al.*, 2017).

Listeria monocytogenes outbreaks have also been reported in South Africa from 2017 to 2018 (Smith *et al.*, 2019). A total of 1 060 cases and 216 deaths were reported from different provinces in South Africa between 2017 and 2018 (Smith *et al.*, 2019). These oubreaks were linked with consumption of polony, sourced from a food processing plant in Polokwane (NICD, 2018), where *L. monocytogenes* was detected in the processing facility (Chersich *et al.*, 2018). Three provinces: Gauteng, Western Cape, and Kwazulu-Natal were reported with the highest cases. Table 3.3 summarises the total number of reported cases and deaths per province.

Table 2. 3 Number of listeriosis cases and deaths, reported between 01 January 2017 and 17 July 2018 in South Africa (Smith et al., 2019)

Provinces	**Number of deaths (% of those with outcome available)**	**Number of cases (% of total cases)**
Gauteng province	108 (28)	614 (58)
Western Cape	32 (24)	136 (13)
KwaZulu-Natal	21 (28)	83 (8)
Limpopo	11 (22)	55 (5)
Eastern Cape	13 (33)	53 (5)
Mpumalanga	11 (23)	48 (5)
Free State	9 (28)	36 (3)
North West	8 (30)	29 (3)
Northern Cape	3 (50)	6 (1)
Total	**216 (27)**	**1060**

ANTIMICROBIAL RESISTANCE

Antimicrobial resistance (AMR) is an alarming health issue, trending worldwide (Thanner *et al.*, 2016). Antibiotics are used worldwide in clinics to fight against bacterial infections in humans and animals, as well as in agricultural operations to promote growth (Fang *et al.*, 2019). However, the rapid utilisation, improper use or overuse of antibiotic has resulted in bacteria resisting the actions of antimicrobial drugs used in humans and animals (Lerma *et al.*, 2014). Microorganisms can develop resistance to certain classes of antibiotics through chromosomal genes mutation (Partridge, 2015). However, resistance in *Enterobacteriaceae* mostly occur as a result of mobile resistance genes which are captured by various mobile genetic elements and then transfer them to plasmids (Partridge, 2015). Resistance genes carried in plasmids can then be transferred between cells of different bacteria and species (horizontal transfer of genes), and can also be tansferred during cell division (vertical transfer of genes) (Partridge, 2015).

Gram-negative bacteria have been reported to increasingly becoming resistant to multiple antibiotics making the choice of antimicrobial drugs difficult (Oliphant & Eroschenko, 2015a). This has therefore become a major concern, because Gram-negative bacteria are predominantly implicated in many outbreaks linked with fresh produce consumption (Blaak *et al.*, 2014; Vital *et al.*, 2017). Hence, humans may get antimicrobial resistant bacteria through fresh produce consumption (Blaak *et al.*, 2014; Van Hoek *et al.*, 2015). Once antimicrobial resistance develops in an animal or humans' guts, resistance genes can be disserminated onto the environmment through faecal matters, contaminating surface, waters and soils (Said *et al.*, 2015; Thanner *et al.*, 2016). The resistance genes may then be transferred to the bacteria found in the environment. Through contaminated irrigation water, inadequately treated manure and soils, resistant microorganisms can be transferred to crops and may remain on crops until they are consumed (Schwaiger *et al.*, 2011; Van Hoek *et al.*, 2015; Hölzel *et al.*, 2018).

Some antimicrobial classes used in health cares are such as: penicillins, cephalosporins, carbapenems, aminoglycosides, vancomycin, tetracyclines, fluoroquinolones and sulfonamides (Oliphant & Eroschenko, 2015b). These antimicrobial agents have different mechanisms in which they act against bacteria. For instance, some agents like penicillins and cephalosporins are β-lactams which counteract bacterial infections by inhibiting the synthesis of bacterial cell wall (Oliphant & Eroschenko, 2015b). However, bacteria like the *Enterobacteriaceae* produce the β-lactamase enzyme which renders β-lactams inactive (Oliphant & Eroschenko, 2015a). The *Enterobacteriaceae* has emerged with resistance to penicillin and the broad-spectrum cephalosphorines, as a results

of the extended-spectrum β-lactamases (ESBLs) production, this has therefore become a threat globally (Pitout & Laupland, 2008; Blaak *et al.*, 2014; Zurfluh *et al.*, 2015).

Extended Spectrum β-lactamases (ESBLs) producing Enterobacteriaceae

Extended Spectrum β-lactamase is an enzyme which confers bacterial resistance, by hydrolysing the β-lactam ring, thereby rendering the β-lactams inactive (Pitout & Laupland, 2008). The ESBLs have the ability of hydrolysing penicillin, 1st-,2nd-,3rd-and the 4th generation cephalosporins and monobactams, consequently, impacting bacterial infections treatment and control in humans (Ojer-Usoz *et al.*, 2013). However, the EBSLs are inhibited by clavulanic acids (Blaak *et al.*, 2014). In addition, the ESBL-producing strains do not confer resistance to the cephamycins, and carbapenems (Pitout & Laupland, 2008). ESBL-producing organisms are widely disseminated in the environment, and have been found in water (Diab *et al.*, 2018), as well in animal products (Ojer-Usoz *et al.*, 2013). Contaminated environments, irrigation water, soils and improperly composted manure may transfer the ESBL-producing bacteria to the fresh produce (Zurfluh *et al.*, 2015). Contaminated fresh produce may represent a route of human exposure to the ESBLs-producing bacteria (Van Hoek *et al.*, 2015). The dissemination of resistant bacteria between irrigation water and fresh produce, can be related to a study done in South Africa by (Du Plessis *et al.*, 2015) which has indicated the transfer of bacteria from river water used for irrigation to the irrigated onions.

Extended spectrum β-lactamases-producing *Enterobacteriaceae* has been isolated from vegetables (Blaak *et al.*, 2014). In a study done by Zurfluh *et al.* (2015) in Switzerland, on vegetables imported from the Dominican Republic, India, Thailand and Vietnam, 25% isolates were found with one or more ESBL-producing *Enterobacteriaceae*, and 78% of them were identified with multidrug resistance. van Hoek *et al.*, (2015) has reported on the prevalence of the 3rd generation cephalosporins (3GC) resistant *Enterobacteriaceae* on some vegetables bought from Dutch stores. There are four groups of ESBLs that are frequently reported in clinical isolation, namely: TEM, SHV, OXA, and CTX-M; with CTX-M being the most common type of ESBLs described (Pitout & Laupland, 2008; Ojer-Usoz *et al.*, 2013; Shaikh *et al.*, 2015; Zurfluh *et al.*, 2015). There are many different types of CTX-M β-lactamases however, they have been divided into five subgroups (CTX-M1, CTX-M2, CTX-M9, and CTX-M25) due to the amino-acid sequencing alignment (Pitout & Laupland, 2016). The isolates from *Escherichia coli* and *Klebsiella pneumonia* have been reported as the predominant ESBL-producing organisms globally (Pitout & Laupland, 2008 van Hoek *et al.*, 2015). Zurfluh *et al.* (2015), have obtained 60 ESBLs producing *Enterobacteriaceae* isolates, and 52 of the isolates were *E. coli* and *K. pneumonia* accounting for 26 isolates each.

However, other *Enterobacteriaceae* species such as *Citrobacter* spp, *Enterobacter* spp., *Kluyvera*, *Serratia* and *Rahnella* also carry the ESBL genes, and are found in agricultural soils, animal manure and faecal contaminated water (Blaak *et al.*, 2014; Van Hoek *et al.*, 2015) thereby standing a chance of transferring the ESBL genes to the fresh produce. Most of the acquired β-lactamases main origin is not known, however, the *bla*$_{CTX-M}$ genes have been reported to have originated from some species of genus *Kluyvera*, which is naturally found in the soil, and it is hardly implicated with human infections (D'Andrea *et al.*, 2013).

SOURCES OF MICROBIAL CONTAMINATION ON FRESH PRODUCE

Fresh produce can be at risk of contamination with microorganisms before harvest, during harvest and after harvest until it reaches the consumer (Brackett, 1999). Contamination may occur in different ways, either from humans, animal or environmental sources (Mir *et al.*, 2018). At the pre-harvest level, fresh produce may get contaminated through contaminated soils, wild animals' droppings, irrigation water, and biosolids/manure (Septembre-malaterre *et al.*, 2018). Whereas after harvesting, the processing operations may expose fresh produce to contamination (Mir *et al.*, 2018). Contamination during processing of fresh produce could be from contaminated wash water, workers hands, processing machinery and unhygienic packaging materials, (Qadri *et al.*, 2015; Diseases, 2018; Mir *et al.*, 2018). Moreover, transportation, distribution, and handling of fresh produce at the retail point of sale can also contribute to contamination of the products (Thunberg *et al.*, 2002).

Pre-harvest contamination sources of fresh produce

Soil as a source of fresh contamination

The soil in which fresh produce is grown is considered as an important source of microbial pathogens at pre-harvest level. Microorganisms such as *Clostridium perfrigens, Bacillus cereus, C. botulinum,* and *L. monocytogenes*, exist naturally in the soil and can be picked up by fresh produce planted in such soils (Sant'Ana *et al.*, 2014; Alegbeleye *et al.*, 2018). However, other microorganisms can be introduced to the soil through untreated contaminated animal manure, contaminated irrigation water, and wild animals faecal matters (Sant'Ana *et al.*, 2014; Rajwar *et al.*, 2016). Some microorganisms may then survive and grow in the soils, depending on parameters such as environmental temperature, the soil type, moisture, soil pH prevalence of competitors, the binding ability of bacteria to the soil, as well as the intensity of contamination (Jacobsen & Bech, 2012; Sant'Ana *et al.*, 2014).

A study done by Underthun *et al.* (2018) on the survival of *Salmonella* and *Escherichia coli* in Candler sand (CS) and Orangeburg sandy loam (OSL) at different moisture levels and temperatures, found that *Salmonella* and *E. coli* in Candler sand

exposed at 30°C could survive for up to 168 and 56 days respectively, and could also survive for 168 and 224 days in Orangeburg sandy loam exposed to 30°C. The availability of nutrients in the soil enables the growth of pathogens (Sant'Ana *et al.*, 2014). Contaminated soil may transfer pathogens to fresh produce when it gets into contact with the edible parts of the produce, which is also facilitated by raindrop splashes (Sant'Ana *et al.*, 2014; Rajwar *et al.*, 2016). In addition, germinating seeds may also acquire pathogens from contaminated soils, resulting in bacteria attaching on roots and edible parts of the crop (Sant'Ana *et al.*, 2014).

Irrigation water as a source of contamination

Water is a significant requirement for crop production; however, in some parts of the world, rainfall is seasonal and sometimes not sufficient for growing crops. However groundwater, wastewater and surface water from rivers, lakes, and ponds are used for irrigation as an alternative source, allowing maximum production throughout the year (Sant'Ana *et al.*, 2014; Rajwar *et al.*, 2016; Alegbeleye *et al.*, 2018). Nonetheless, this water used for irrigation has been identified in many studies as a transmission source of food-borne pathogens to fresh produce (Jung *et al.*, 2014; Allende & Monaghan, 2015; Alegbeleye *et al.*, 2018). The microbiological quality of surface water used for irrigation might be compromised due to animals defecating in water, contaminated soils and sewage materials (Sant'Ana *et al.*, 2014; Rajwar *et al.*, 2016).

In many developing countries, potable water for irrigation is scarce, hence many opt to use untreated surface water for irrigation (Jung *et al.*, 2014). In South Africa, surface water and groundwater are commonly used for irrigation. Unfortunately, some surface waters have been regarded unfit for agricultural purposes due to high *E. coli* counts exceeding the allowable counts (1 000 CFU.100 mL^{-1}) set by the Department of Water and Sanitation as well as the World Health Organization (WHO) (Du Plessis & Korsten, 2015). In a study done by Britz *et al.* (2012) on Irrigation water quality and food safety, Western Cape river water was reported with low microbial quality. Similarly, the river water used for irrigation in Sobantu, South Africa, was reported with poor microbiological quality (Gemmell & Schmidt, 2012). As the population grows, informal settlements are also expanding, and in most cases, informal settlements are associated with poor services, little or no sanitation facilities, therefore, this could be one of the factors contributing to contamination of surface water (Du Plessis & Korsten, 2015; Jongman and Korsten, 2018). In addition, Du Plessis & Korsten, (2015) have identified storm-water services, improper functioning of wastewater treatment plants, as well as intensified urbanisation, as factors contributing to surface water contaminations.

Contaminated irrigation water may transfer human pathogens to fresh produce, imposing risks to human health through consumption of contaminated fresh produce, especially those minimally processed (Olaniran *et al.*, 2009; Gemmell & Schmidt, 2012; Antwi-Agyei *et al.*, 2015). Lettuce and onions have been implicated with Hepatitis A outbreak, which was transferred from sewage-contaminated irrigation water (Alegbeleye *et al.*, 2018). There has also been a reported outbreak of salmonellosis linked with tomatoes irrigated with contaminated water from a pond (Jung *et al.*, 2014). The ability of irrigation water to transfer pathogens onto fresh produce was also proven in a study done by Lapidot & Yaron (2016), in which water contaminated with *Salmonella typhimurium* was used to drip irrigate mature parsley plants, and the pathogen was also detected on parsley plants that was regrown three weeks later after harvesting. Similarly, a study conducted by Decol *et al.* (2017) has confirmed irrigation water as a major risk factor for fresh produce contamination at pre-harvest level. The transfer of human pathogens from contaminated irrigation water onto vegetables is influenced by certain factors such as the method of irrigation, the source of irrigation water, the type of crop being irrigated and the intensity of the pathogen in water (Alegbeleye *et al.*, 2018).

Manure and biosolids as a source of contamination

Some pathogens such as *Salmonella*, *Escherichia coli and Campylobacter jejuni*, are naturally found in the intestinal tract of many animals including humans (Monaghan, 2010; Olaimat & Holley, 2012). Some parasites (*Cryptosporidium*, *Giardia*, and *Cyclospora*) and viruses (norovirus and Hepatitis A) are also carried in human and animals' gut and shed in faeces in a large number (Glass *et al.*, 2009; Monaghan, 2010; Dixon, 2016). In many countries organic materials are considered good for improving properties for crop production soils, therefore many farmers consider using livestock excreta, slurries, abattoir wastes and biosolids to amend their crop production soils (Alegbeleye *et al.*, 2018). The use of animal manure and biosolids might be used more by many farmers as soil fertiliser for agricultural purposes because it is more affordable than commercial fertilisers (Jacobsen & Bech, 2012; Sant'Ana *et al.*, 2014; Alegbeleye *et al.*, 2018). However, untreated manure and contaminated biosolids may pose risks of contaminating fresh vegetables, water resources through runoff, as well as soils with enteric pathogens, which may further spread widely in the farming environment, consequently exacerbating contamination risks (Jung *et al.*, 2014; Sant'Ana *et al.*, 2014; Alegbeleye *et al.*, 2018). It is therefore advisable to properly treat animal manure, biosolids and slurries before applying them onto crop fields (Rajwar *et al.*, 2016). Practicing appropriate storage and processing methods of manure, which include

anaerobic digestion, aeration of sludge, and composting can lower the pathogen populations in contaminated manure (Suslow *et al.*, 2003).

Wild animals and insects

Most fresh produce is cultivated in areas accessible to wild animals (birds, reptiles, rodents, amphibians, and certain helminths) and insects (flies and beetles) (Sant'Ana *et al.*, 2014). These wild animals and insects may get into contact with crops thereby transmitting pathogens to the fresh produce (Alegbeleye *et al.*, 2018). Some studies have identified *E. coli*, *Salmonella*, as well as *Campylobacter* as pathogens carried by some birds including chickens (Alegbeleye *et al.*, 2018). Insects as vectors for food-borne pathogens could be a serious problem because they are widely found in cultivation fields, and it is very difficult to restrict their contact with produce (Alegbeleye *et al.*, 2018). An observation done under laboratory conditions has indicated direct transmission of bacteria from contaminated flies to plant leaves or fruits (Suslow *et al.*, 2003; Berger *et al.*, 2010; Alegbeleye *et al.*, 2018). Wild animals and insects do not only contaminate fresh produce through direct contact, but they may serve as pathogen vehicles to water sources, soil, and manure, thereby contaminating the produce indirectly (Sant'Ana *et al.*, 2014).

Sources of contamination at harvest

Harvesting is done either manually or mechanically. Both of these methods imply direct contact between surfaces/handlers and fresh produce which could result in direct transmission of food-borne pathogens to the produce (Brackett, 1999; Li *et al.*, 2015). Cross contaminations could occur between fresh produce and workers' hands or material used during harvesting such as gloves, knives, buckets, and harvesting equipment (Matthews, 2013; Jung *et al.*, 2014; Li *et al.*, 2015). Verhaelen *et al.* (2013) has observed the transfer of human norovirus from produce to gloves and from gloves to produce. Studies have also observed the accumulation of pathogens on fruit pickers' hands which had the potential of further contaminating fruits (Li *et al.*, 2015). In addition, not all farm workers know or ensure proper personal hygiene, therefore such workers could transmit food-borne pathogens during fresh produce handling (Brackett, 1999).

During harvesting, inedible leaves/parts of produce are removed, and this practice might result in tissue damage, and further handling may encourage the transfer and penetration of pathogens into the vegetable/fruit through the cut edge, and microbial growth (Sela & Fallik, 2009; Jung *et al.*, 2014). The knife used for trimming might also be contaminated therefore, it can serve as a vehicle for food-borne pathogens (Jung *et al.*, 2014). Machinery used for harvesting may get contaminated with food-borne pathogens from

soils and manure, consequently introducing these contaminations to the produce being harvested (Jung *et al.*, 2014). To reduce risks of contaminations, thoroughly cleaning and sanitation of harvesting machines and tools should be exercised regularly (Matthews, 2013; Jung *et al.*, 2014). Washing of equipment is essential for removing the soils and other debris which could be contaminated, though it will not reduce the number of microorganisms. Therefore it is crucial to always involve a sanitation step in order to reduce the microbial load, and this should be applied to all tools used during harvesting (Matthews, 2013; Jung *et al.*, 2014; Sant'Ana *et al.*, 2014; Li *et al.*, 2015). It is also very important to offer training to workers on good personal hygiene, for instance, washing their hands always before and after handling the produce.

Post-harvest contamination sources

After harvesting, fresh produce goes through several unit operations to make sure that it reaches the consumers in an acceptable, consistent, and preferred form. These operations may involve transport from the field to the packing house, processing, storage, distribution and transportation (Li *et al.*, 2015; Rajwar *et al.*, 2016). However, these operations may compromise fresh produce safety through contact between the fresh produce and tools/equipment, workers and wash water; that may present high possibilities of contracting pathogenic contamination (Gil *et al.*, 2015; Rajwar *et al.*, 2016).

During storage or transportation of harvested produce from the farm to the packing house, contaminated surfaces and improper handling can compromise the quality of fresh produce (Gil *et al.*, 2015). Placing crates for carrying the produce onto the soil might lead to contamination from the soil to the produce during loading in the transport (Gil *et al.*, 2015). Fresh produce transported in a truck that was used to carry other products or a truck that is not frequently cleaned, can potentially expose fresh produce in transit to contaminations(Brackett, 1999). In addition, transporting fresh produce together with other products such as raw meat is not advisable, as it can cause cross contaminations (Brackett, 1999). Moreover, fresh produce can get bruised during transportation, allowing the release of growth nutrients consequently promoting bacterial growth (Gil *et al.*, 2015). It is therefore important to carry and transport the produce in an appropriate manner, and in a vehicle that will lower contamination levels and fresh produce bruises (EFSA, 2014; Gil *et al.*, 2015).

Processing of fresh produce includes cutting/shredding, washing, sanitation, packaging and storing (Jung *et al.*, 2014). Cutting/shredding of vegetable/fruits or trimming off inedible parts, opens up the fresh produce tissues, making it easier for microorganism invasion (Qadri *et al.*, 2015). This also results in the release of nutrient exudates which in combination with favourable temperatures facilitate microbial growth (Gil *et al.*, 2015). Risks

of contaminations can also be induced by workers who lack the knowledge on good hygiene practices (GHP) (Castro-Ibáñez *et al.*, 2017). Contaminated workers' hands and surface can also contaminate fresh produce during processing. An increase in pathogen population has been observed on fresh produce after cutting and shredding (Waitt *et al.*, 2013; Jung *et al.*, 2014). Washing of fresh produce during processing is very crucial as it removes soils, debris and microorganisms from fresh produce (Francis *et al.*, 2012; Zhou *et al.*, 2014; Murray *et al.*, 2018). However, contaminated wash water can potentially contaminate fresh produce (Maffei *et al.*, 2017). Therefore, potable water supplemented with sanitizers is used in many food processing industries to reduce microbial load from the produce (Francis *et al.*, 2012; Matthews, 2013). However, the wash water quality and the effectiveness of the sanitizer can be affected by the accumulation of soils, debris and juices from fresh produce (Zhou *et al.*, 2014). As a result, microorganisms can accumulate in the wash water and cross contaminate fresh produce (Zhou *et al.*, 2014). This allows packaging of contaminated produce, which then may put consumer's health at risk. There has been a reported outbreak linked with cabbage that was washed with contaminated underground water in Korea (Li *et al.*, 2015).

The wash water containing a sanitizing agent may effectively remove microorganism from fresh produce (Gil *et al.*, 2015). However, contamination could still occur through contaminated surfaces, workers' hands and packaging materials (EFSA, 2014; Jung *et al.*, 2014; Castro-Ibáñez *et al.*, 2017). The conditions to which fresh produce is exposed during packaging, distribution, storage, or market shelves can encourage the proliferation and survival of microorganisms on the produce (Brackett, 1999; Castro-Ibáñez *et al.*, 2017). Sant'Ana *et al.* (2012) have observed the growth potential of *Salmonella* and *L. monocytogenes* on vegetables stored at high temperatures of 15^{o}C and 25^{o}C (Jung *et al.*, 2014). High temperatures during transportation of fresh produce can also be experienced. Refrigerated trailers with improper air circulation may cause improper cooling, due to accumulation of heat released from product as respiration and that absorbed from outside the trailer, may lead to temperatures favourable for microbial growth (Brackett, 1999; Gil *et al.*, 2015).

Fresh produce requires appropriate handling and temperature exposure at any point along the production chain to avoid contaminations and bacterial growth. Figure 2.1 summarises factors contaminate fresh produce.

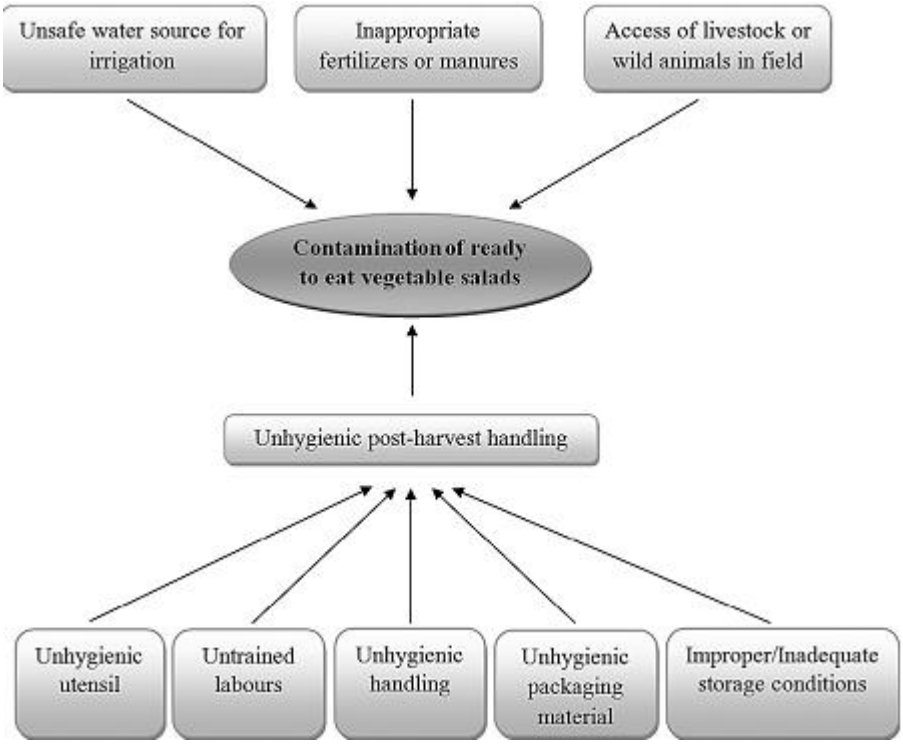

Figure 2.1 systematic diagram representing route of ready to eat vegetables contaminations (Mir *et al.*, 2018)

ENTRY AND ESTABLISHMENT OF MICROORGANISMS ON FRESH PRODUCE

Attachment

For a certain bacteria to colonise fresh produce, it has to be in contact with the fresh produce surface and attach to it (Alegbeleye *et al.*, 2018). Different microorganism serovars may attach differently on fresh produce due to the serovars' distinct properties (Patel & Sharma, 2010). There are factors that aid in the attachment of pathogens on fresh produce such as: motility of pathogens, the interaction between organisms and the discharge of nutrients from the plant (Olaimat & Holley, 2012). Motility of pathogens enables entry of pathogens into fresh produce through stomata, lenticels, broken trichomes, and bruises or cracks found on the produce surfaces (Olaimat & Holley, 2012; Alegbeleye *et al.*, 2018). Studies have reported the colonisation of intact lettuce, basil and spinach by *enterotoxigenic E. coli* facilitated by the fimbriae attachment (Olaimat & Holley, 2012). The ability of pathogens to move around the external surfaces of produce renders them chances of entry into produce wounds leading to pathogens accessing nutrients that will allow them to grow to reach the effective dose, and further spread on fresh produce (Olaimat & Holley, 2012; Sant'Ana *et al.*, 2014). The attachment of pathogens onto fresh produce is also influenced by the type of the crop involved (Patel & Sharma, 2010; Alegbeleye *et al.*, 2018).

Internalisation

Pathogens that successfully attach on the produce surface may penetrate the interior of the fresh produce (Sant'Ana *et al.*, 2014; Alegbeleye *et al.*, 2018). Microorganisms may penetrate the plant through the plant roots or seeds and can move to the edible parts of the plant (Alegbeleye *et al.*, 2018). Most bacteria gain entry through plant openings such as the stomata, lenticels, and bruises (Deering *et al.*, 2012). Once the bacteria penetrate, they then move to the internal tissues with the help of water, or by flagella motility, or the chemotaxis process (Golberg *et al.*, 2011; Deering *et al.*, 2012). However, internalisation is influence by factors such as: the pathogen's mechanism of entry, the type of the plant and age, the root morphology, the biology and type of soil, and the type of bacteria attached to the produce (Deering *et al.*, 2012; Sant'Ana *et al.*, 2014; Alegbeleye *et al.*, 2018). Internalisation of pathogens in different fresh produce has been shown in several studies (Golberg *et al.*, 2011; Deering *et al.*, 2012). *Escherichia coli* O157:H7 from contaminated radish seeds was observed on the surfaces of radish sprouts and penetrated the interior parts during sprout growth (Olaimat & Holley, 2012). However, in a study that was done by Zhang *et al.*, (2009) on internalisation of *E. coli* O157:H7 in lettuce, a lack of internalisation was observed in all tested samples.

SURVIVAL OF MICROORGANISMS ON FRESH PRODUCE

Survival of microorganisms on fresh produce may pose health risks to consumers. The formation of biofilms on plant surfaces is one of the factors that advocate for bacteria survival on fresh produce (Lamas *et al.*, 2018). A group of bacterial cells may collectively form on fresh produce in exopolysaccharide materials to protect the bacteria cells from environmental stresses (Olaimat & Holley, 2012). Several studies have observed the formation of biofilms on leafy vegetables such as spinach, lettuce and cabbage (Elhariry, 2011; Ng *et al.*, 2017). Biofilms help bacteria to resist sanitising agents making it difficult to minimise contamination in the processing environments (Grande Burgos *et al.*, 2017; Adator *et al.*, 2018).

Microorganisms survival in soils and on produce can also be influenced by other factors such as nutrient accessibility, toxic compounds released by plant, temperature, soil type, exposure to ultraviolet light, protozoan predation and the preliminary number of existing microorganisms (Jacobsen & Bech, 2012; Olaimat & Holley, 2012; Overbeek, 2014). Microorganisms such as *Salmonella*, *E. coli* O157:H7, *L. monocytogenes* have been reported to persist and survive on fresh produce for a number of days (Olaimat & Holley, 2012). Guévremont *et al.* (2015) have indicated the ability of microorganisms to survive on fresh produce for a long period of time. In a study that was done by Gupta & Madramootoo

(2017) on *Escherichia coli* contamination on ready-to-eat lettuce, bacteria were found to have had survived in the soil and on lettuce leaves for more than 30 days after inoculation.

Survival and growth of microorganisms is also influenced by different temperatures, giving them the ability to contaminate other refrigerated foods. Some bacteria can survive and grow at refrigeration temperatures (Lakicevic *et al.*, 2015). *Listeria monocytogenes* is a common example of bacteria that can survive at temperatures as low as 0º to 4ºC (Smith *et al.*, 2018). Studies have observed the survival and growth of *L. monocytogenes* on produce refrigerated at 4ºC (Parish *et al.*, 2003). Nonetheless, *L. monocytogenes* can be destroyed by temperatures as high as 70ºC (Monaghan, 2010). *Escherichia coli* and *Salmonella* may survive at 4ºC, but their growth is inhibited (Parish *et al.*, 2003). In a study done on *E. coli* O157:H7 in bovine faeces, *E. coli* O157:H7 had survived for a period of 42-49 days at 37ºC, and 49-56 days at 22ºC (Beuchat, 2002). Another study has reported *E. coli* O157:H7 survival on lettuce contaminated by manure, for a period of 15 days at 4ºC storage temperature (Beuchat, 2002).

The pH of fresh produce can also limit or enhance the growth and survival of microorganisms. Some vegetables are found in the pH range that supports the growth of pathogenic microorganisms, however, the pH range for some fresh produce such as fully ripe tomatoes may inhibit the growth of pathogens (Beuchat, 2002). High pH, especially on bruised fresh produce may support the survival and growth of bacteria, however, yeast and moulds may grow at low pH (Beuchat, 2002). *Listeria monocytogenes* grows at the pH ranging from 4.1 to 9.6 (Smith *et al.*, 2018). However *L. monocytogenes* can survive at the pH levels high as pH 12 (Liu *et al.*, 2005).

INTERVENTION METHODS TO ENSURE MICROBIAL SAFETY OF FRESH PRODUCE

Food-borne illnesses linked with contaminated fresh produce have been reported globally (Wadamori *et al.*, 2017; Murray *et al.*, 2018). It is evident in many publications that food-borne pathogens can potentially contaminate fresh produce at any stage from production and throughout the supply chain to the consumer's table (Abong *et al.*, 2008; Shenge *et al.*, 2015; Van Dyk *et al*, 2016; De Bruin *et al.*, 2016). However, there are several control or treatment methods that can help to mitigate the population of microorganisms on fresh produce. The first significant method is to prevent contaminations, but it might be impractical in some scenarios (Parish *et al.*, 2003; Ramos *et al.*, 2013). Therefore to ensure minimal microbial load on fresh produce, disinfection methods which entail chemical use, are widely used in fresh produce industries (Castro-Ibáñez *et al.*, 2017).

Prevention

Prevention of contamination results in minimal pathogens on fresh produce, subsequently minimising food-borne outbreaks (Ramos *et al.*, 2013). For effective results, prevention should begin in the field. Good Agricultural Practices (GAP) and Good Handling Practices (GHP) are applied as pre-requisite to food safety management strategies, in preventing the spread of food-borne pathogens (Van Boxstael *et al.*, 2013; Castro-Ibáñez *et al.*, 2017). Possible contaminants in the cultivation field should be identified and solutions or control measures to reduce or prevent them from spreading should be applied (Castro-Ibáñez *et al.*, 2017). Some fields may have been exposed to livestock and wild animals that constitute the ability of contaminating the field, or may have been previously flooded or were fertilised with improperly composted manure containing high number of microorganisms (Alegbeleye *et al.*, 2018). Studies have indicated irrigation water as a source of crop contaminations at the production level (Brackett, 1999; Pachepsky *et al.*, 2011; Johannessen *et al.*, 2015), therefore to prevent contaminations, irrigation water should be regularly tested for microorganisms, and treatments such as the use of chlorine, peracetic acid, hydrogen peroxide and other treatments can be applied (Alegbeleye *et al.*, 2018). Standard enforcement policies and sanitation training must be provided to workers in dealing with fresh produce, and they should be advised to comply with the standards and hygienic practices (Castro-Ibáñez *et al.*, 2017).

Fresh produce disinfection

There are several disinfection methods used on fresh produce, including chemical-based methods, physical-based methods, and natural or biological-based methods (Goodburn & Wallace, 2013; Meireles *et al.*, 2016). Chemical-based methods are the most common methods used in fresh produce industry in wash water (Abadias *et al.*, 2011; Shen *et al.*, 2012; Goodburn & Wallace, 2013). Chemical-based methods include the use of chlorine compounds, calcium lactate, chlorine dioxide, copper compounds, electrolyzed oxidising water, hydrogen peroxide, ozone, quaternary ammonium, sodium bicarbonate, and weak organic acids, and many others (Parish *et al.*, 2003; Goodburn & Wallace, 2013; Meireles *et al.*, 2016). Chemicals can be used in combination with other treatments methods, or as a free standing chemical, to kill microorganisms on fresh produce (Sela & Fallik, 2009; Feliziani *et al.*, 2016; Castro-Ibáñez *et al.*, 2017). The degree to which these chemicals reduce microorganism population depends on the nature of microorganisms, the initial microbial load, the surfaces to be washed, pathogens internalisation in fresh produce, the temperature and the length of exposure to a certain disinfectant (Castro-Ibáñez *et al.*, 2017).

Regardless of a number of chemicals available for disinfecting fresh produce, chlorine has been a popular disinfectant used in fresh produce industries (Abadias *et al.*, 2011).

Chlorine

Chlorine is an oxidising agent which can be accessed in a solid form (calcium hypochlorite), an aqueous solution (sodium hypochlorite) and as chlorine gas (Parish *et al.*, 2003; Feliziani *et al.*, 2016). The application of chlorine is done by dissolving chlorine in water in which fresh produce is immersed, or used for spraying vegetable as a washing method; it is then followed by rinsing with potable water, in order to wash off residual chemicals and by-products (Li *et al.*, 2015; Castro-Ibáñez *et al.*, 2017). A chlorine concentration varying from 50 to 200 ppm with a contact time ranging from 1 to 5 minutes is used for sanitising fresh produce (Parish *et al.*, 2003; Goodburn & Wallace, 2013; Ramos *et al.*, 2013; Castro-Ibáñez *et al.*, 2017). The pH (preferably pH 6-7) and the organic load of wash water must be measured and maintained at ideal levels for the effectiveness of chlorine disinfection (Li *et al.*, 2015). Studies have indicated the ability of chlorine to reduce microbial load by a value ranging from <1 log $CFU.g^{-1}$ to > 3.15 log $CFU.g^{-1}$, considering the inoculation method used, chlorine concentration, exposure time and the type of bacteria being disinfected (Ramos *et al.*, 2013). Beuchat *et al.* (2001) have evaluated the efficacy of chlorine treatment against pathogens inoculated on tomatoes; a concentration of 200 ppm was used, and ≥3.07 and ≥3.33 $\log_{10}$ reduction on *Salmonella* and *L. monocytogenes* respectively was observed.

Chlorine (Cl) is cost effective, easy to access, and does not alter the nutritional and sensory qualities of the fresh produce (Parish *et al.*, 2003; Castro-Ibáñez *et al.*, 2017). However, chlorine may interact with organic matter from the soil or debris, and leafy tissues (Shen *et al.*, 2016), then produce disinfection by-product (trihalomethanes, haloacetic acids, haloactonitriles, halonitromethanes and many others) which then inactivate or reduce the activity of chlorine disinfection, consequently allowing the proliferation of microorganisms on fresh produce even after processing (Ruiz-Cruz *et al.*, 2007; Murray *et al.*, 2018; Stefán *et al.*, 2019). The exposure of chlorinated disinfection by-products (DBPs) to humans may present harmful health effects that they might be toxic or carcinogenic (Parish *et al.*, 2003; Muellner *et al.*, 2007; Castro-Ibáñez *et al.*, 2017). In a simulated washing study done by Lee *et al.* (2019) lettuce, cabbage and strawberries were tested for DBPs before washing and after washing, and results were that DBPs were found in unwashed produce in low levels, and the washing had significantly increased the formation and concentration of DBPs on fresh produce, however, the formation of DBPs on fresh produce is influenced by the type of produce.

The use of chlorine in water has however been a concern globally, therefore, several alternative disinfection strategies have been introduced (the use of chlorine dioxide, ozone, copper compound, ionising irradiation, ultraviolet light, bacteriophages, and bacteriocins) to replace the use of chlorine (Meireles *et al.*, 2016). In a study done by Abadias *et al.* (2011), peroxyacetic acid also known as peracetic acid (PAA), hydrogen peroxide and N-acetyl-L-cysteine were suggested as potential alternative replacements for chlorine. Neo *et al.* (2013) has found PAA more effective than Cl on reducing pathogens on mung beans, hence, suggested peracetic acid as a potential alternative replacement for chlorine. However, Ruiz-Cruz *et al.* (2007) have found Cl more effective than PA in lowering pathogens, and have also reported that the efficacy of Cl was reduced by the presence of organic matter, which does not affect the PA's efficacy. Acidified sodium chlorite (ASC) was found more effective compared to chlorine and PA, on reducing *E. coli* O157:H7, *L. monocytogenes,* and *Salmonella* from fresh-cut carrots to undetectable levels at all concentrations used (Ruiz-Cruz *et al.*, 2007). However, the highest ASC concentration (1 000 ppm) used was found to have affected the quality of shredded carrots (Ruiz-Cruz *et al.*, 2007). Peracetic acid activity is not affected by the presence of organic matter, its pH remains stable unlike chlorine, and PAA has not been identified with harmful chlorinated by products (Neo *et al.*, 2013), hence, the most suggested potential alternative disinfectant for chlorine on fresh produce wash water.

CONCLUSIONS

The significant role fresh produce plays in human health is undeniable. Fresh produce (Fresh fruits and vegetables) are rich sources of minerals, vitamins and fibre which offer protection against cardiovascular diseases, diabetes, and cancer (Faour-Klingbeil *et al.*, 2016). Therefore, the increased consumption of fresh produce may results in a healthy population, with less heart and lung diseases, as well as less obese people (Boeing *et al.*, 2012). Apart from the health benefits, fresh produce is also associated with economic benefits, and South Africa is one of the countries with a big market for fresh produce (Korsten *et al.*, 2015). However, consumption of contaminated fresh produce presents a great risk of food-borne illness. Literature has indicated fresh produce as a potential transfer vehicle of microbial pathogens to humans. Many illness outbreaks linked with fresh produce have been reported worldwide (Franz *et al.*, 2018). A number of deaths linked with these outbreaks have also been reported, and this has therefore raised a global concern around fresh produce safety globally (Fao/Who, 2008; Jung *et al.*, 2014).

Reviews have highlighted leafy vegetables and fresh cut salads as the frequent cause of fresh produce related outbreaks (Murray *et al.*, 2018). Contamination of fresh

produce with pathogens can originate at any point either pre- or postharvest (Korir *et al.*, 2016). Most fresh produce is eaten raw without heat treatment that could eliminate microorganisms, this has however been perceived as a major problem, owing to the potential transmission of food pathogens from fresh produce to humans (Olaimat and Holley, 2012). Once fresh produce gets contaminated with pathogens, it becomes difficult to eradicate pathogens in the absence of thermal treatment. This is such a risk to the public's health, because the pathogens that are frequently associated with fresh produce have been reported to increasingly becoming resistant to many antibiotics, (Vital *et al.*, 2017). Through consumption of contaminated fresh produce, humans may acquire antimicrobial resistant bacteria which may interfere with clinical treatments (Blaak *et al.*, 2014). The treatment normally employed to inactivate microorganisms from fresh produce in many produce industries is by washing with chemical disinfectants, of which chlorine is mostly used (Parish *et al.*, 2003). However, studies have indicated that chlorinated washing may not completely remove all microorganisms from fresh produce, due to factors like internalisation of microorganisms, the type of organism involved and the initial microbial load on the produce, the surface of the produce involved, and the formation of disinfection by-products (DBPs) (Ruiz-Cruz *et al.*, 2007; Castro-Ibáñez *et al.*, 2017).

The prevalence of microorganisms on fresh produce has been reported worldwide, as seen in literature (Shenge *et al.*, 2015; Ssemanda *et al.*, 2017). Literature has also indicated the persistence of microorganisms on fresh produce sold in different districts of South Africa. Pathogenic *E. coli* O157:H7 was detected on some fresh produce samples collected from Omathole district in the Eastern Cape, of with some isolates were resistant to a few of the antibiotics used (Abong *et al.*, 2008). Although it was only a few samples that were positive with *E. coli* O157:H7 and resistant to some antibiotics, these results still raise a concern on fresh produce safety, because during handling and possible exposure to high temperatures, the microbiological quality of fresh produce could be compromised (Matthews, 2013). Also, resistant genes could be disseminated to the environment or other areas by those eating contaminated raw fresh produce (Van Hoek *et al.*, 2015). During fresh produce processing, cross contaminations can happen between contact surfaces and fresh produce this was demonstrated in a study done in Gauteng and Northwest province of South Africa, in which *Salmonella Typhimurium* was isolated from fresh produce samples obtained from the processing facilities (De Bruin *et al.*, 2016). The presence of microorganisms on fresh produce at pre-harvest level and post-harvest level has been reported in a few studies conducted in South Africa (Van Dyk *et al.*, 2016; Du Plessis *et al.*, 2017). Hence, the microbiological quality of fresh produce sold in South Africa is still a concern. Moreover, these studies were not done in the Western Cape; hence, there is limited information

available regarding the microbiological quality of fresh produce sold in the Western Cape markets.

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CHAPTER 3

EVALUATION OF MICROBIAL INDICATORS FROM SELECTED FRESH PRODUCE COLLECTED WITHIN THE PACKHOUSE

ABSTRACT

Microorganisms of the *Enterobacteriaceae* family have been frequently implicated with food-borne outbreaks related to consumption of fresh produce. Fresh produce consumption has increased in many countries, as a result of the health benefits it provides. This study aimed at enumerating microorganism indicators: *E. coli*, coliforms and *Enterobacteriaceae* from fresh produce sampled before and after pack-house processing steps, in order to determine the impact of processing on the microbial load of the produce. Samples were collected from a pack-house situated in Phillippi, Western Cape, South Africa. A total of 45 broccoli stem, 45 carrot, 18 red cabbage, and 75 lettuce samples were collected. *Enterobacteriaceae* was recovered from all broccoli stem, carrot and cabbage samples in average levels ranging from 2.1 to 5.13 log CFU.g^{-1}. Coliforms were also recovered from all samples in average counts ranging from 1.62 to 4.81 log CFU.g^{-1}. *Escherichia coli* were only found on a few samples: 2 of 45 (4%) untreated broccoli stem samples, 1 of 45 (2%) untreated carrot samples, and 6 of 45 (13%) shredded carrot samples. No *E. coli* was detected on cabbage samples. *E. coli* ranged from < 1 log CFU.g^{-1} (undetected) to 2.035 log CFU.g^{-1}. Samples peeled and washed in chlorine (150 - 200 ppm) water had significantly lower average counts than unwashed samples. The reduction levels of *Enterobacteriaceae* and coliform observed in this study ranged from 0. 94 to 1.17 log CFU.g^{-1} and 0.83 to 0.95 log CFU.g^{-1} respectively on broccoli and carrot samples. On lettuce samples, *Enterobacteriaceae* and coliform reduction ranged from 0.89 to 2.35 CFU.g^{-1} and from 0.69 to 2.27 CFU.g^{-1}, respectively. An increase in microorganisms was observed in shredded samples. The average levels of coliforms on shredded samples did not comply with the previous South African Department of Health guideline limits (2.3 CFU.g^{-1}) (under review) for ready to eat fresh produce. Therefore, this study identified shredding and packaging as potential contamination points.

INTRODUCTION

In many countries including South Africa, the fresh produce industry has grown, due to the increased demand for fresh fruits and vegetables (De Bruin *et al.*, 2016; Ssemanda *et al.*, 2017). Incorporation of fresh produce into the diet has become a norm, due to the notion that increased consumption of fruits and vegetables counteract chances of certain illnesses and promote good health (Olaimat & Holley, 2012; Septembre-Malaterre *et al.*, 2018). In many countries including South Africa, the fresh produce industry is well established. With emerging developments and lifestyles, consumers are demanding more minimally processed fresh produce that has only been washed, trimmed/ peeled or shredded (Nguz *et al.*, 2005). However, food-borne illness outbreaks attributed to minimally processed/raw fresh produce like pre-packaged lettuce, salads, sprouts and other leafy vegetables, have increased globally (Jung *et al.*, 2014; Wadamori *et al.*, 2017; Murray *et al.*, 2018). Therefore, microbiological quality and safety of fresh produce eaten raw has become a concern (Qadri *et al.*, 2015).

Many studies have identified irrigation water as a contributing factor to fresh produce contamination at pre-harvest level (Pachepsky *et al.*, 2011; Allende and Monaghan, 2015; Faour-Klingbeil *et al.*, 2016; Rajwar *et al.*, 2016; Alegbeleye *et al.*, 2018). Some South African rivers used for fresh produce irrigation are of poor microbial quality (Korsten *et al.*, 2015; Jongman and Korsten, 2018). According to Britz *et al.* (2012) microbial transmission from contaminated irrigation water to fresh produce is highly likely. Nonetheless, it is not only pre-harvest factors that contribute to fresh produce contaminations. The processing steps to which fresh produce are subjected after harvest can also serve as a route for contamination (Rajwar *et al.*, 2016). Poor hygiene practices can potentially transfer microorganisms onto fresh produce. Wash water, packaging material, processing equipment, surfaces as well as handlers hands may contaminate fresh produce (Gil *et al.*, 2015; Murray *et al.*, 2018). It is therefore crucial to monitor microbial populations on fresh produce in order to verify the microbial safety of the product (Castro-Ibáñez *et al.*, 2017).

Most of the reported outbreaks linked with consumption of fresh produce have been attributed to members of the *Enterobacteriaceae* (Newman *et al.*, 2017). *Enterobacteriaceae* represents a large group of Gram-negative bacteria, both pathogenic and non-pathogenic, including coliforms and non-coliforms, as well as *E. coli* (Hervert *et al.*, 2016; Osaili *et al.*, 2018). In the food industry, *Escherichia coli* is used as indicator of faecal contamination, and also as an indication of the possible presence of pathogenic *E. coli* (Leclerc *et al.*, 2001; Korir *et al.*, 2016). Coliforms and *Enterobacteriaceae* are used to evaluate hygiene, and post-processing contamination (Eden, 2014). *Enterobacteriaceae* is widely distributed in many environments such as marine, soils and vegetables, and can be found on fresh

produce in a large number (NSW Food Authority, 2009). However, during processing of fresh produce, washing of fresh produce is intended to reduce microorganisms to lower levels. However, during handling of fresh produce, further contamination is likely to occur through workers hands, surfaces or processing equipment (Rajwar *et al.*, 2016). An increase in levels of microbial indicators (*E. coli*, coliforms, and *Enterobacteriaceae)* on fresh produce in the processing chain can be an indicator of unhygienic conditions within the processing unit. Therefore, testing for microorganisms on fresh produce collected from different sites in the processing chain may reveal potential contamination points (De Bruin *et al.*, 2016). Studies done on the microbial content of fresh produce from farm to market have observed higher levels of microbial indicators on the produce after processing, and at the market level, than on produce sourced directly from the field (Shenge *et al.*, 2015; Ssemanda *et al.*, 2017). This is an indication that further contamination may occur during fresh produce processing and may put consumers' health at risk. In South Africa, information on the prevalence of microbial indicators on fresh produce at different stages of the supply chain is limited. Therefore, the aim of this study was to enumerate *E. coli*, coliforms and *Enterobacteriaceae* from fresh produce samples before and after pack-house processing, in order to determine the impact of processing on the microbial load of the produce.

MATERIALS AND METHODS

Site and samples selection

This study focused on determination of the microbial quality of fresh produce collected within the pack-house. A pack-house situated in Philippi, Western Cape, South Africa was selected for this study. The selected pack-house has a Food Safety Audit certificate, and it supplies the processed produce to a national retail chain. In addition, this pack-house receives a range of fresh produce from various farms that are in possession of Global GAP certificates. (Anonymous (*confidentiality), 2019, Food Safety Management representative, Fresh produce Pack-house, Western Cape, South Africa, personal communication, 4 August). Fresh produce selected in this study was broccoli coleslaw (consisted of shredded broccoli stems, carrots, and cabbage) and butter lettuce. These fresh produce types were selected because they are often consumed raw in salads without heat treatment.

All the fresh produce received at the pack-house goes through several processing steps. The end products are supplied to formal markets, as well as informal markets within the pack-house vicinity. Broccoli, cabbage, and carrots are used as ingredients for fresh cut-mix bags that are supplied to the retailers as coleslaw mix. Lettuce is supplied to the market as pre-packed lettuce heads and to local customers as loose lettuce. Both pre-packed and

loose lettuce should be washed before use by the customer. Lettuce is also supplied in Ready-to-Eat pillow-packs. All three types of lettuce products were included in this study.

Sampling design

A total of 45 broccoli stem, 45 carrot, 18 cabbage, and 75 different lettuce samples were collected. Samples of each produce were collected from different processing steps in line with individual ingredient processing steps. The processing steps and sampling points (SP) are summarised in Figure 3.1 and Table 3.1 (Broccoli coleslaw), and Figure 3.2 and Table 3.2 (Lettuce).

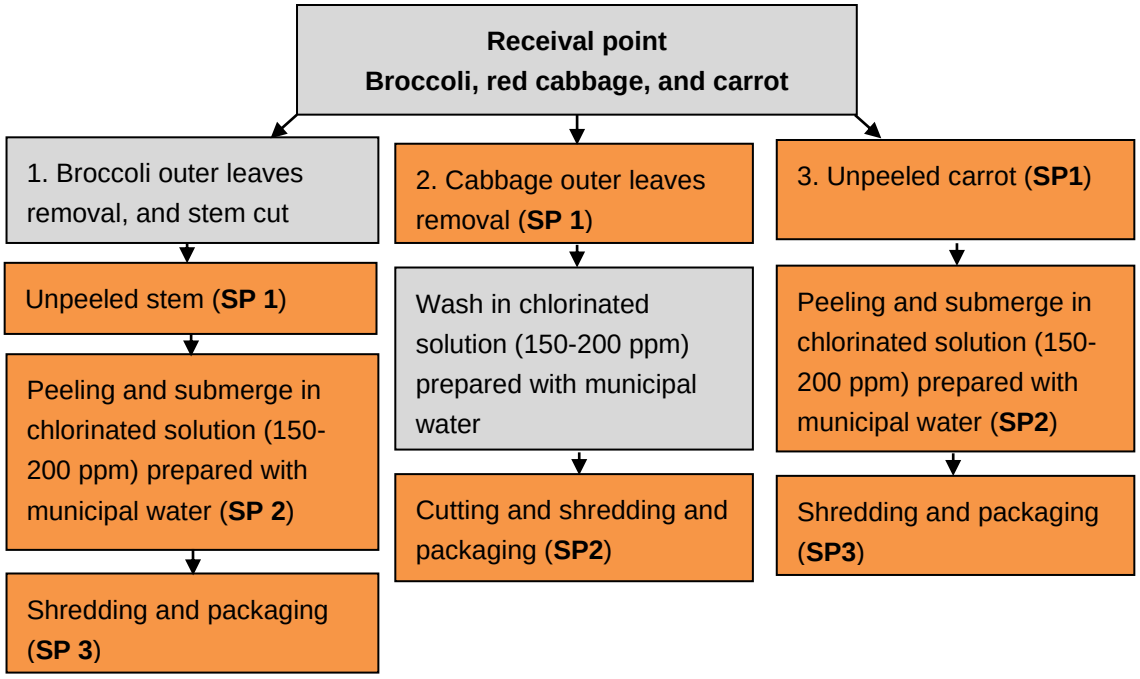

Figure 3. 1 Broccoli coleslaw ingredients processing steps and sampling points (SP)

Table 3. 1 Description of terms in the processing steps and sampling points in Figure 3.1

Terms	SP	Definitions
Untreated broccoli stem	SP1	Broccoli stem that is not peeled or washed.
Treated broccoli stem	SP2	Broccoli stem that is peeled and submerged in chlorine solution (150–200 ppm) prepared with municipal water, for at least a minute.
Untreated cabbage	SP1	Unwashed cabbage with outer leaves removed.
Untreated carrot	SP1	Carrot that is not washed or peeled
Treated carrots	SP2	Peeled carrots submerged in chlorine solution (150–200 ppm) prepared with municipal water, for at least a minute.

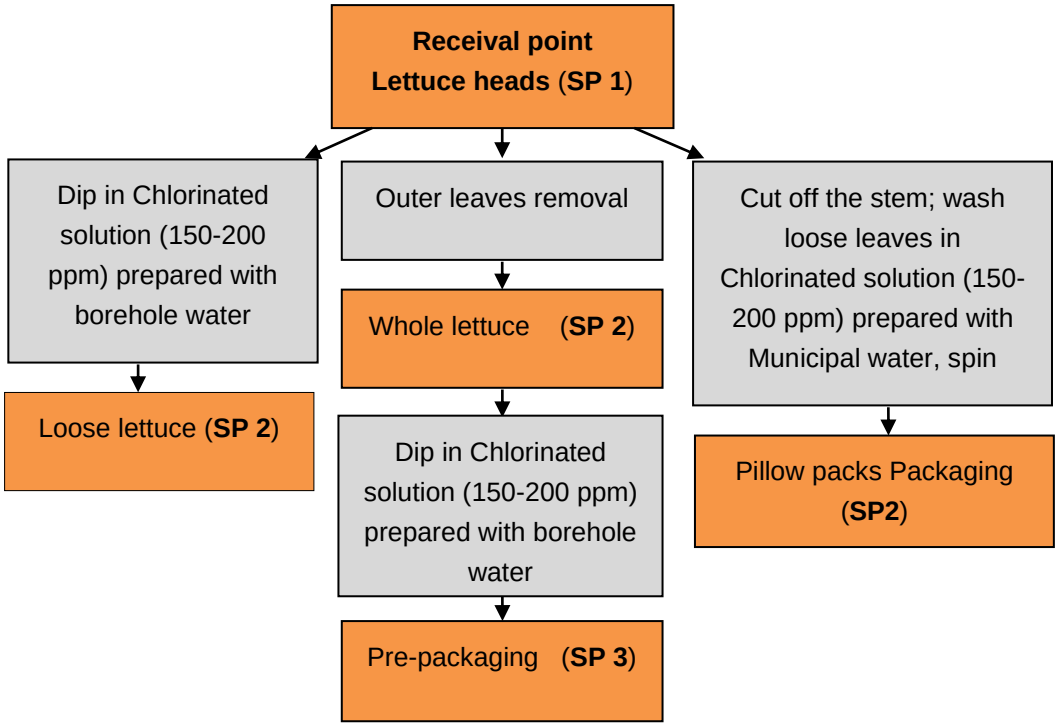

Figure 3. 2 Lettuce processing steps and sampling points (SP)

Table 3. 2 Description of terms in the processing steps and sampling points in Figure 3.2

Terms	SP	Definition
Lettuce head	SP 1	Unprocessed lettuce
Loose Lettuce	SP 2	Loose lettuce head dipped in chlorinated (150-200 ppm) borehole water for at least a minute
Whole lettuce	SP 2	Unwashed lettuce head with outer leaves removed
Pre-packs	SP 3	Whole lettuce dipped in chlorinated solution (150-200 ppm) prepared with borehole water, for at least one minute; packaged and labelled as wash before use.
Pillow-packs	SP 2	Packaged ready to eat lettuce leaves.

Chlorine solution (150-200 ppm) prepared with municipal water was used to wash the produce prepared for ready to eat packs (shredded samples and pillow packs). Whereas chlorinated solution (150-200 ppm) prepared with borehole water was used to wash the produce that requires further washing before use by the consumer. All samples were collected in triplicate from each sampling point, and this was repeated five times, in different weeks. Upon sampling, samples were packed in sealable plastic bags and put in a cooler

box with crushed ice, then transported to the Department of Food Science, Stellenbosch University. Upon arrival, samples were stored at 4^{o}C until analysed, which was done within 24 hours.

MICROBIOLOGICAL ANALYSIS

Research study design

To determine the potential contamination points along the processing chain of fresh produce, samples collected from the different processing points were all analysed for three hygiene indicators: *Enterobacteriaceae,* coliforms and *E. coli.* Unprocessed samples (untreated carrot, untreated cabbage and untreated broccoli stem) that were not washed were used to indicate the initial microbial load on fresh produce samples. An increase or reduction on initial counts of the indicators (*Enterobacteriaceae*, coliforms, and *E. coli*) after washing was used to determine the potential contamination points. All samples were tested at the Department of Food Science, Stellenbosch University. To minimise the risk of further contamination, samples were handled under aseptic conditions in the laboratory.

Sample preparation

From each sampling point (SP) indicated in Figures 3.1 and 3.2, three samples were collected. These samples were then prepared to form a composite sample as follows: each sample was cut in half, and each of these halves was further cut into smaller pieces on a sterile metal tray, with a sterile knife. A 100 g was then collected from each tray, and combined on a separate sterile tray to form a 300 g composite sample. From the composite sample, three triplicate 25 g samples were collected (as shown in Figure 3.3) into three separate sterile polyethylene stomacher bags.

Thereafter, 225 mL of 0.1% buffered peptone water (BPW) (Merck, South Africa) was added to the bags containing 25 g samples and stomached at 230 rpm for 2 min in a 220V Interscience Bag Mixer.

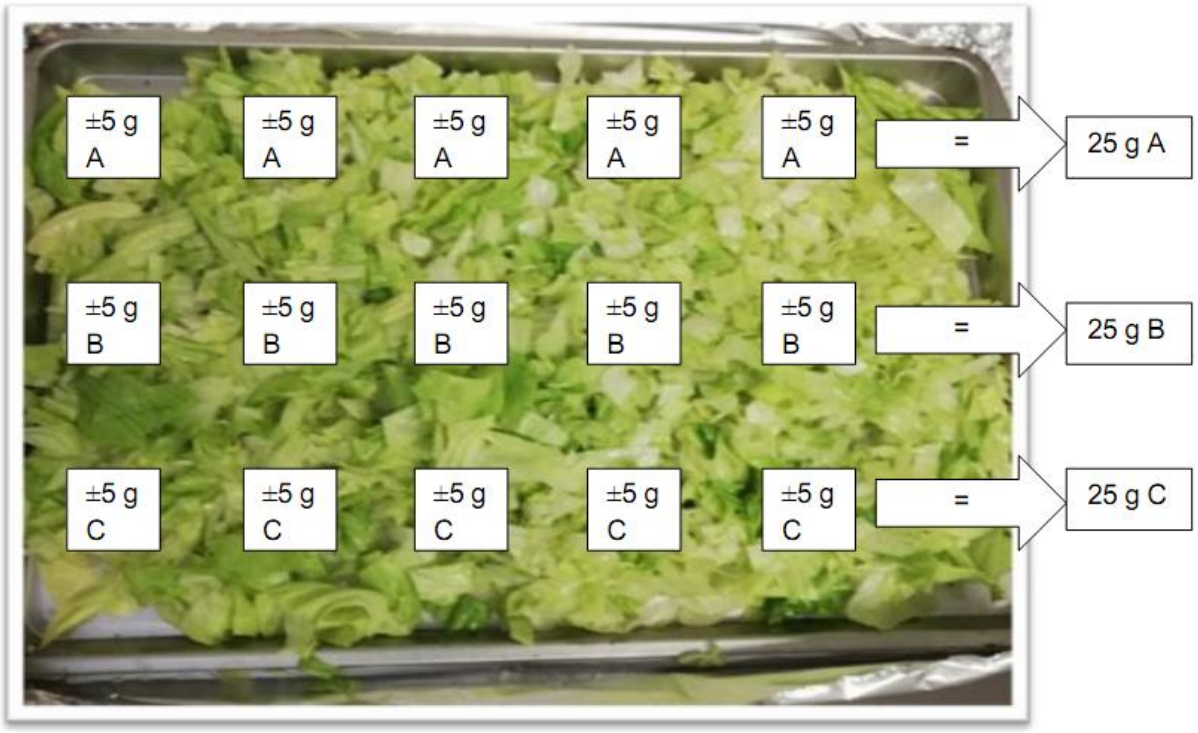

Figure 3.3 Illustrations of how 25 g of each produce samples were sampled in triplicate (A, B, and C) from the 300 g composite sample.

Enumeration of *E. coli,* Coliforms, and *Enterobacteriaceae*

Serial dilutions were prepared ranging from 10^{-2} – 10^{-5} by transferring 1 mL of the homogenised sample to 9 mL of Ringer solution according to SANS 6887-3:2004 method. This was followed by plating out each dilution in duplicate, using the standard aseptic pour plate method. Enumeration *of E. coli* and coliforms was done by plating out on Rapid *E. coli* 2 agar (Bio-Rad, South Africa) following the SANS 4832:2006 method. Enumeration of *Enterobacteriaceae* was done by plating out onto Violet Red Bile Glucose Agar (VRBGA) (Oxoid, South Africa) according to the SANS 21528-2:2005 method. Plates were then incubated at 37 ± 2ºC for 24 h. After incubation, coliforms and *E. coli,* as well as *Enterobacteriaceae* colonies were identified and counted on plates that had counts of 10 - 300 colonies. *Escherichia coli* growth was identified by violet to pink colonies, and coliforms growth was identified by blue to green colonies on Rapid E. coli 2 Agar (Figure 3.4 A) (Bio-Rad, 2013). *Enterobacteriaceae* growth was identified by pink to red/purple colonies surrounded or not surrounded by a halo (Figure 3.4B) (SANS 21528-2:2005).

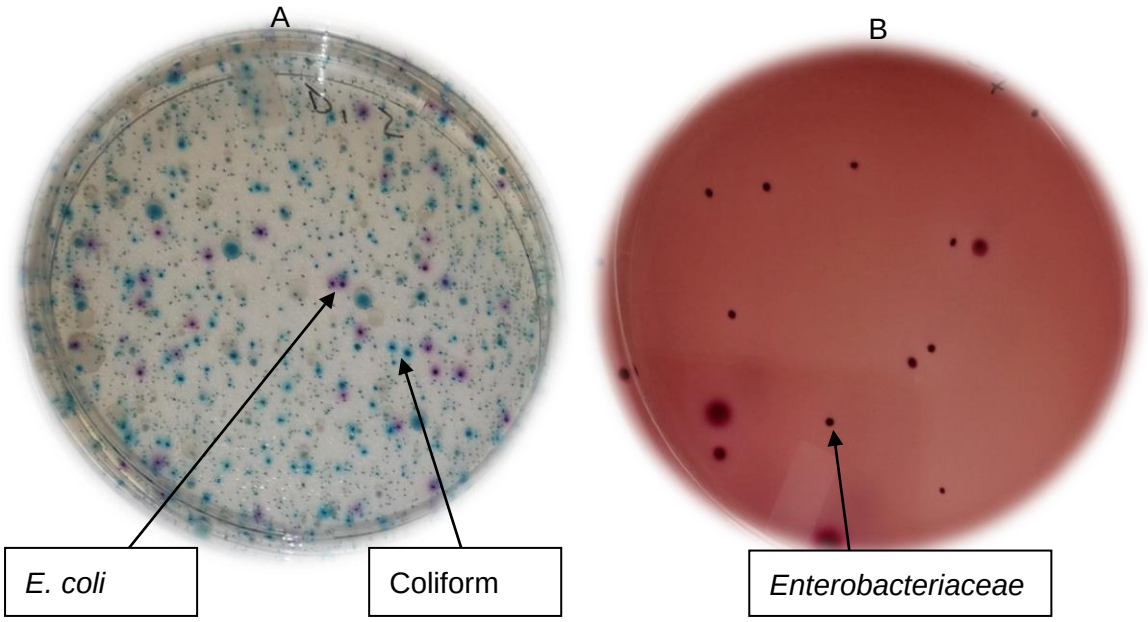

Figure 3.4 Identification of (A) *E. coli* and Coliform growth on RAPID' *E. coli* 2 agar; and (B) *Enterobacteriaceae* on Violet Red Bile Glucose (VRBG) agar

STATISTICAL ANALYSIS

Colony counts were converted to log values (log CFU.g^{-1}) prior to analysis. Sigma Plot version 13 software was used to calculate and plot the mean values and standard deviations. One-way ANOVA's were conducted to compare average measurements between treatments. For post hoc testing, Fisher Least Significant Difference testing was done. Homogeneity of variance was tested using Levene's test, and for the cases where this hypothesis was rejected, the Welsh test was done with Games-Howell post hoc testing. The P-value ($P<0.05$) was used to determine the statistical significance, at 95% confidence interval (Prof. Martin Kidd, 2019. Centre of Statistical Analysis, Department of statistic, Stellenbosch University, personal communication, 27 September).

RESULTS AND DISCUSSION

The microbiological changes on selected fresh produce along the processing chain were successfully determined, and results obtained are indicated in Figures 3.5, 3.6, 3.7 and 3.8. To indicate the limitation of the colony counting method, zero counts were recorded as <1 log CFU.g^{-1}. The lowest detection level is indicated with a black horizontal line in all graphs (Figures 3.5, 3.6, 3.7, and 3.8).

Prevalence of microbial indicator: Enterobacteriaceae, coliforms and E. coli on broccoli samples

Figure 3.5 indicates the average counts of microorganisms detected on broccoli samples. The average counts of *Enterobacteriaceae* (3.04 CFU.g^{-1}) and coliforms (2.57 CFU.g^{-1}) on untreated (not washed and not peeled) broccoli stem samples were significantly ($P<0.05$) reduced to 2.1 log CFU.g^{-1} and 1.62 log CFU.g^{-1}, respectively, after peeling and washing in chlorinated solution (150- 200 ppm) (see treated sample in Fig. 3.5).

The level of coliforms and *E. coli* on treated broccoli samples (peeled and washed in chlorine solution (150-200 ppm) falls within the DoH guideline limits (under review) (< 2.3 log CFU.g^{-1} for coliform and 0 CFU.g^{-1} for *E. coli* (DoH, 2002). The European Commission guidelines (EC, 2007) suggest satisfactory E*. coli* levels up to 2.0 log CFU.g^{-1} on ready to eat fresh fruits and vegetables. Concerning *Enterobacteriaceae*, there is no standard guideline limiting its level on fresh produce.

Results in Fig. 3.5 also indicate that after shredding both *Enterobacteriaceae* and coliform average counts on samples have significantly ($P<0.05$) increased to 3.28 and 2.85 CFU.g^{-1} respectively. Coliform levels on shredded samples were higher than the DoH guideline limits (2.3 log CFU.g^{-1}) (DoH, 2002). *Escherichia coli* was detected on untreated (unwashed, and not peeled) broccoli stem samples in low levels (average counts 1.05 log CFU.g^{-1}), however, it was not recovered on treated (washed and peeled) or shredded samples.

It is evident from literature that washing produce may not completely eliminate microorganisms, but it can reduce microorganism loads to a certain level (Luo *et al.*, 2012; Goodburn & Wallace, 2013). The bacterial reduction during washing with chlorinated solution observed in many studies ranged from <1 log CFU.g^{-1} to 3.15 log CFU.g^{-1}, depending on the contact time and the disinfection concentration used (Ramos *et al.*, 2013). Results obtained in this study are therefore in line with literature findings. Ssemanda *et al.* (2017) in Rwanda have observed an average microbial reduction of 2.1 log CFU.g^{-1} after peeling, trimming and washing (with or without sanitizers) of fresh vegetables that had the initial microorganism populations ranging from 4.6 to 6.3 log CFU.g^{-1}. Gu *et al.* (2018) have observed a reduction of 1.33 ± 0.54 log CFU.g^{-1} after washing spinach that had an average microbial population of 6.12 ± 0.61 log CFU.g^{-1}. Although these two studies indicated higher microbial reduction levels than results obtained in this study, it also confirmed that washing methods do not completely remove microorganisms from fresh produce.

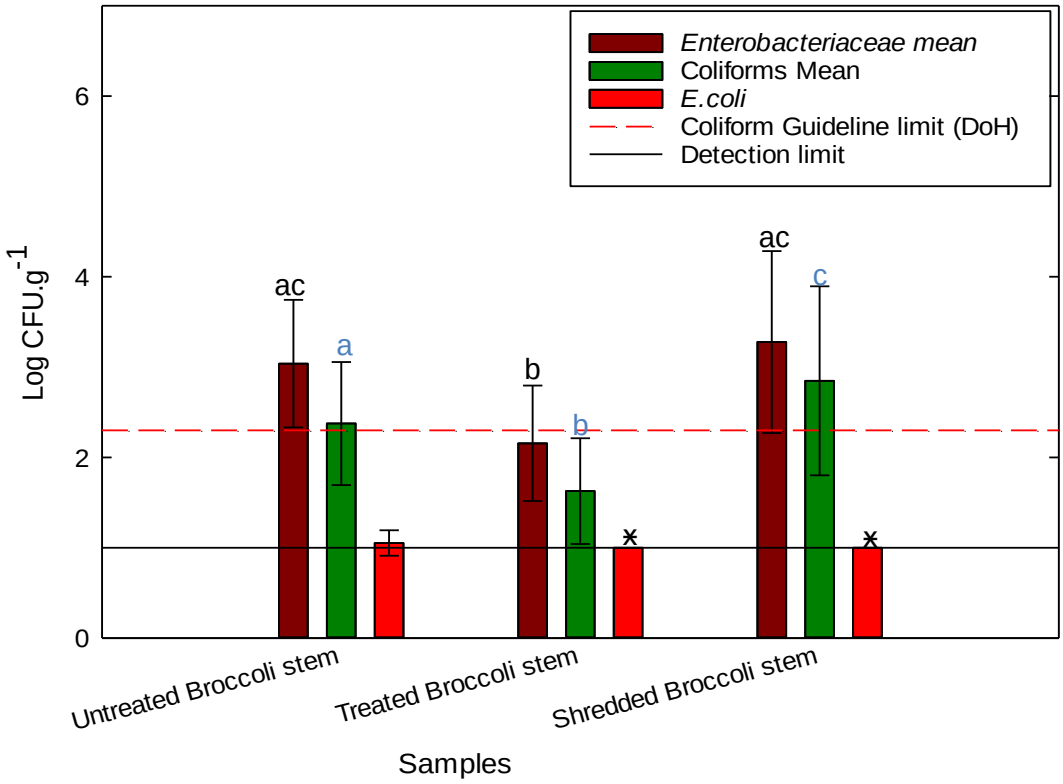

Figure 3. 5 Average levels of *Enterobacteriaceae*, coliforms and *E.coli* on broccoli stem samples, and the significant differences. Bars with different letters indicate average counts that are significantly different at 95% confidence level (P<0.05). Bars with the same letters indicate average counts that are not significantly different. Black letters represent *Enterobacteriaceae*; blue letters represent coliforms. ⁎=not detected. The error bars indicate standard deviation. The red dotted line indicates the highest accepted level of coliforms on ready to eat fresh produce, by the South African department of Health (DoH) (DoH, 2002). The black line indicates the lowest level at which microorganisms could be detected in this study (1 log CFU^{-1}). Untreated = unwashed produce. Treated = washed in chlorinated solution.

Prevalence of microbial indicators: Enterobacteriaceae, coliforms and E. coli on Carrot Samples

Of all the produce tested, the highest average levels of microorganisms were recovered from carrot samples; ranging from 3.29 to 5.13 log CFU.g^{-1} and from 3.07 to 4.81 log CFU.g^{-1} for *Enterobacteriaceae* and coliforms, respectively (Figure 3.6). *Escherichia coli* were recovered from untreated (unwashed and unpeeled) samples at low levels (1.02 log CFU.g^{-1}), and from shredded samples at levels (2.03 log CFU.g^{-1}) higher than the untreated samples. No *E. coli* was detected on treated (washed and peeled) samples. This does not necessarily imply complete absence of *E. coli*, as it could be present at undetectable levels (levels below 1 log CFU^{-1}).

The level of *Enterobacteriaceae* (4.46 log CFU.g^{-1}) on the untreated (unwashed and unpeeled) samples was significantly ($P<0.05$) reduced to 3.29 log CFU.g^{-1} after treatment (washing and peeling) (Figure 3.6). However, after shredding *Enterobacteriaceae* levels had significantly ($P<0.05$) increased to 5.13 log CFU.g^{-1}. The level of *Enterobacteriaceae* (5.13 log CFU.g^{-1}) on shredded carrot samples was significantly ($P<0.05$) higher than *Enterobacteriaceae* level (4.46 log CFU.g^{-1}) on untreated (unwashed and unpeeled) carrot samples. As with *Enterobacteriaceae*, the level of coliform (3.9 CFU.g^{-1}) on untreated carrots was also significantly ($P<0.05$) reduced to 3.07 CFU.g^{-1} after treatment (washing and peeling) (Figure 3.6). The levels of coliforms had also significantly ($P<0.05$) increased to 4.81 CFU.g^{-1} after shredding.

The average level of *E. coli* (1.03 log CFU.g^{-1}) recovered from untreated carrot samples were reduced to undetectable levels after washing and peeling. However, the average level of *E. coli* of 2.03 log CFU.g^{-1} was recovered after shredding. The European commission (EC, 2007) describes *E. coli* on ready to eat unsatisfactory if one or more of the observed values are greater than 3 log CFU.g^{-1}. In this study more than two shredded carrot samples had *E. coli* counts (3.11, 3.04, 4.20, 4.11 and 4. 18 log CFU.g^{-1}) above 3 log CFU.g^{-1}. Hence, the level of *E. coli* recovered from shredded carrot samples in this study is described as unsatisfactory according to the microbiological criteria stipulated by the European Commission (EC, 2007). This may imply inadequate hygiene practices during shredding.

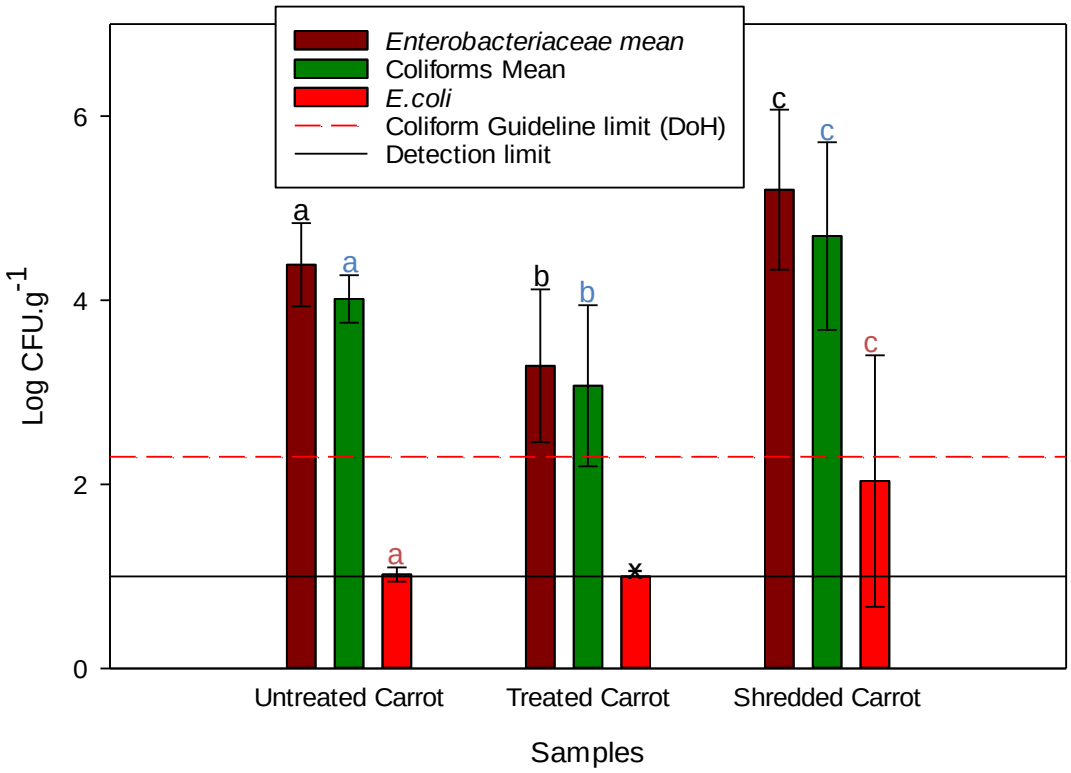

Figure 3. 6 The average levels of *Enterobacteriaceae*, coliforms and *E. coli* on carrot samples and the significant differences. Bars with different letters indicate average counts that are significantly different at 95% confidence level (P<0.05). Bars with the same letters indicate average counts that are not significantly different. Black, blue and red letters represent *Enterobacteriaceae*, coliforms and *E. coli* respectively. ⁎=not detected. The error bars indicate standard deviation. The red dotted line indicates the highest accepted level of coliforms on ready to eat fresh produce, by the South African department of Health (DoH) (DoH, 2002). The black line indicates the lowest level at which microorganisms could be detected in this study (1 log CFU.g^{-1}). Untreated = unwashed produce. Treated = washed in chlorine solution.

Prevalence of microbial indicators: Enterobacteriaceae, coliforms and E. coli on Cabbage Samples

Red cabbage was sampled from only two processing points: (1.) after outer leaf removal (before washing), and (2.) after washing in chlorine solution (150-200 ppm) and shredding. The average levels of *Enterobacteriaceae* and coliforms on red cabbage samples are presented in Figure 3.7. No *E. coli* was detected on cabbage samples. The average levels of *Enterobacteriaceae* and coliforms on untreated (unwashed and unpeeled) (3.12 and 2.69 log CFU.g^{-1}, respectively), and shredded cabbage (3.18 and 2.69 log CFU.g^{-1}, respectively) were similar. However, the coliform levels recovered from shredded samples were still above the current South African DoH guideline limit (≤ 2.3 log CFU.g^{-1}).

Before shredding, the cabbage samples are washed in chlorine solution (150-200 ppm), which is intended to reduce microorganisms on fresh produce to low levels (Goodburn

and Wallace, 2013; Van Haute *et al.*, 2019). Thus, these results still indicate either ineffective disinfection process or re-contamination during shredding.

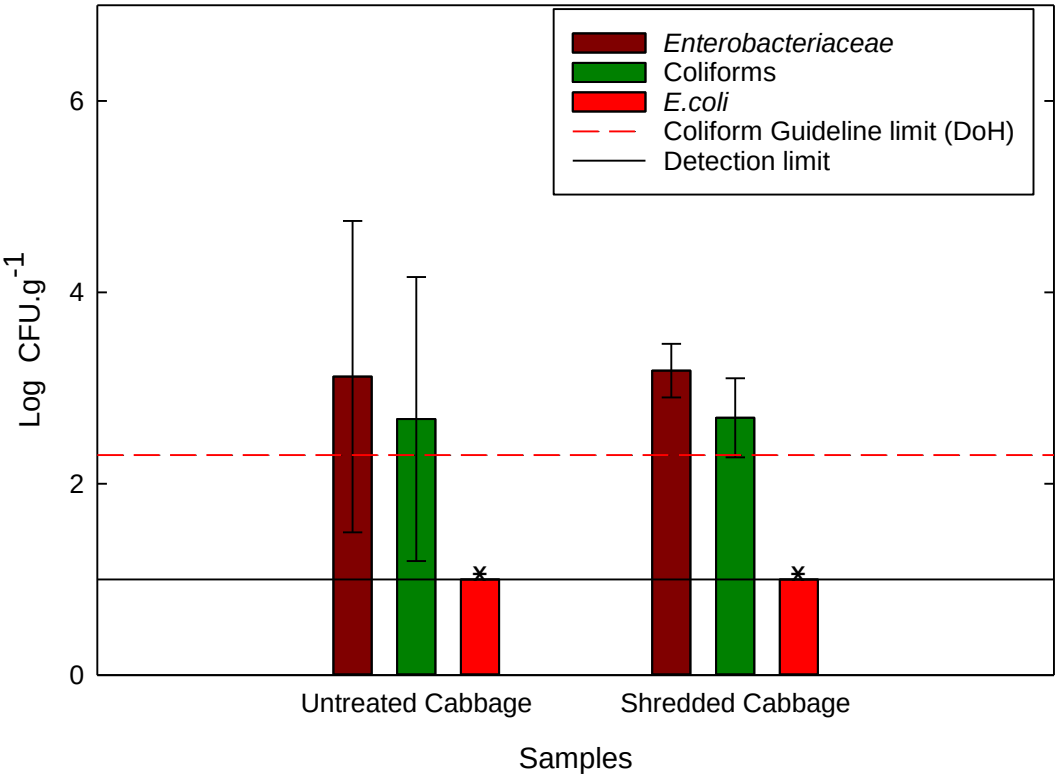

Figure 3.7 The Average levels of *Enterobacteriaceae*, coliform and *E. coli* on cabbage samples. The error bars indicate standard deviation at 95% confident level. ⁎=not detected. The red dotted line indicates the highest accepted level of coliforms on ready to eat fresh produce, by the South African department of Health (DoH) (DoH, 2002). The black line indicates the lowest level at which microorganisms could be detected. Untreated = unwashed produce.

As discussed, the microbiological load of fresh produce samples collected from different processing steps within the pack-house was determined by enumerating three microbial indicators (*Enterobacteriaceae*, coliforms and *E. coli*). *Enterobacteriaceae* was recovered from all samples tested (broccoli stem, carrot, and cabbage) at levels ranging from 2.1 to 5.13 log CFU.g^{-1}. Coliforms were also recovered from all samples tested at levels ranging from 1.62 to 4.81 log CFU.g^{-1}. *Escherichia coli* was detected only on 2 of 45 (4%) untreated broccoli stem samples, 1 of 45 (2%) untreated carrot samples, and shredded carrot samples 6 of 45 (13%). *Escherichia coli* were not recovered from cabbage samples. Overall *E. coli* averages ranged from <1 log CFU.g^{-1} (undetected) to 2.04 log CFU.g^{-1}.

Previous studies have reported similar results. In a study done by Newman *et al.* (2017) on the microbiological load of fresh produce in the United States, *E. coli* was detected on 5 of 11 types of vegetables analysed, in levels ranging from 2.0 – 2.3 log CFU.g^{-1}. In addition, coliforms were prevalent on 10 of 11 types of vegetables analysed in levels ranging

from 1.4 to 3.5 log CFU.g^{-1}. Johnston *et al.* (2005) have also reported *E. coli* and coliforms on leafy greens and herbs at levels ranging from <1 to 1.5 CFU.g^{-1} and <1 to 3.4 log CFU.g^{-1}, respectively. In a study done in Zambia by Nguz *et al.* (2005) on fresh-cut organic vegetables, *E. coli*, coliforms, and *Enterobacteriaceae* were detected at levels ranged from 0.6 to 3 log CFU.g^{-1}, 1.0 to 7.7 log CFU.g^{-1}, and 1.6 to 9.8 log CFU.g^{-1}, respectively. The results of that study were higher than the results obtained in this study.

Enterobacteriaceae and coliforms are widely disseminated in many natural environments, as a result, are highly prevalent on raw vegetables (Colclasure *et al.*, 2015; Osaili *et al.*, 2018). *Escherichia coli* are naturally found in the intestinal tract of warm blooded mammals, including humans, and are disseminated into the environment through faeces (Adam and Mæhlum, 2012). Therefore, the presence of *E. coli* on fresh produce could be indicative of faecal contamination (Julien-Javaux *et al.*, 2019).

Overall carrot samples had the highest average counts of indicator organisms followed by cabbage samples. Broccoli had the lowest microbial loads. In this study, unprocessed broccoli stem and carrot samples carried higher *Enterobacteriaceae* and coliform counts compared to treated samples that were peeled and washed in chlorinated solution (150-200 ppm) water. This is can be expected because untreated samples could be exposed to various possible contamination factors such as contaminated soils, irrigation water, dirt harvesting crates, handling, and transport surfaces (Gemmell and Schmidt, 2012; Rajwar *et al.*, 2016; Alegbeleye *et al.*, 2018).

The average counts of indicator organisms in this study (*Enterobacteriaceae*, coliforms and *E. coli*) were significantly reduced ($p<0.05$) after peeling and washing in chlorine solution at a concentration of 150 - 200 ppm. Microorganisms and most dirt are normally found on the produce surfaces. Therefore, the removal of produce outer layers helps reduce most of the dirt and microorganisms attached to the surface. Washing is a significant step during processing. Washing of fresh produce removes field debris and soils from fresh produce (Murray *et al.*, 2018). The disinfectant agent (e.g. Chlorine) added to the wash water inactivates microorganisms, and is intended to reduce microbial load from fresh produce (Van Haute *et al.*, 2019). Most fresh produce industries add chlorine to the wash water as a disinfecting agent (Abadias *et al.*, 2011). This study's results indicate that the washing of fresh produce in chlorine solution (150-200 ppm) has not completely removed microorganisms from the produce but has only reduced them to slightly lower levels. It has been reported in literature that disinfection (washing) of fresh produce does not completely eradicate microorganisms from fresh produce, but it can reduce microbial population to low levels (Ruiz-Cruz *et al.*, 2007; Neo *et al.*, 2013). The ineffective disinfection could be a result of change on the pH level in chlorine water or the formation of disinfection by-products in the

wash water, which reduce the activity of chlorine (Murray *et al.*, 2018; Stefán *et al.*, 2019). Contact time is also one of the factors affecting the disinfection efficacy. In the fresh produce industry, the contact time ranging between 1- 5 minute is used (Goodburn & Wallace, 2013). Fresh produce sampled in this study was submerged in chlorine water for at least one minute (Anonymous (*confidentiality), 2019, Food Safety Management representative, Fresh produce pack-house, Western Cape, South Africa, personal communication, 4 August).

During shredding and packing, new microorganisms can be introduced to the samples from workers' hands, the shredding machine surfaces and the packaging materials (Mir *et al.*, 2018). Where hygienic conditions have been practiced, the introduction of new contaminations during shredding and packaging might be limited. However, fresh produce have a high water activity (a_w) which supports the growth of microorganisms (Sela & Fallik, 2009). Shredding involves destruction of cell surfaces and the release of nutrients from the produce (Qadri *et al.*, 2015). With high moisture and nutrients, and favourable temperatures, microorganisms remaining after the washing step, are able to multiply and result in the deterioration of the quality of shredded samples (Sela & Fallik, 2009).

Prevalence of microbial indicators: Enterobacteriaceae, coliforms and E. coli on lettuce samples

Figure 3.8 shows the level of *Enterobacteriaceae*, coliforms and *E. coli* recovered from lettuce samples, sampled from different processing steps (Fig. 3.8). Lettuce head was sampled at the point of receipt (unprocessed lettuce head) as it entered the processing plant, with high initial *Enterobacteriaceae* (4.77 log CFU.g^{-1}) and coliform (4.42 log CFU.g^{-1}) loads. Loose lettuce (lettuce head washed and sold as loose lettuce) was prepared by dipping the lettuce head for at least a minute in chlorinated borehole water (150-200 ppm) with the purpose of removing some microorganisms and the field soils the loose lettuce (Anonymous (*confidentiality), 2019. Food Safety Management representative, fresh produce pack-house, Western Cape, South Africa, personal communication, 04 August). Results obtained indicated no significant difference ($p=0.08$) between the average levels of *Enterobacteriaceae* on unprocessed lettuce heads (not washed and no outer leaf removed) (4.77 log CFU.g^{-1}) and loose lettuce (washed lettuce head) (4.44 log CFUg^{-1}). The average coliform levels on unprocessed lettuce head (4.42 log CFU.g^{-1}) and loose lettuce heads (3.68 log CFU.g^{-1}) was also not significantly different ($p=0.17$). On both unprocessed lettuce head and loose lettuce head, no *E. coli* was detected.

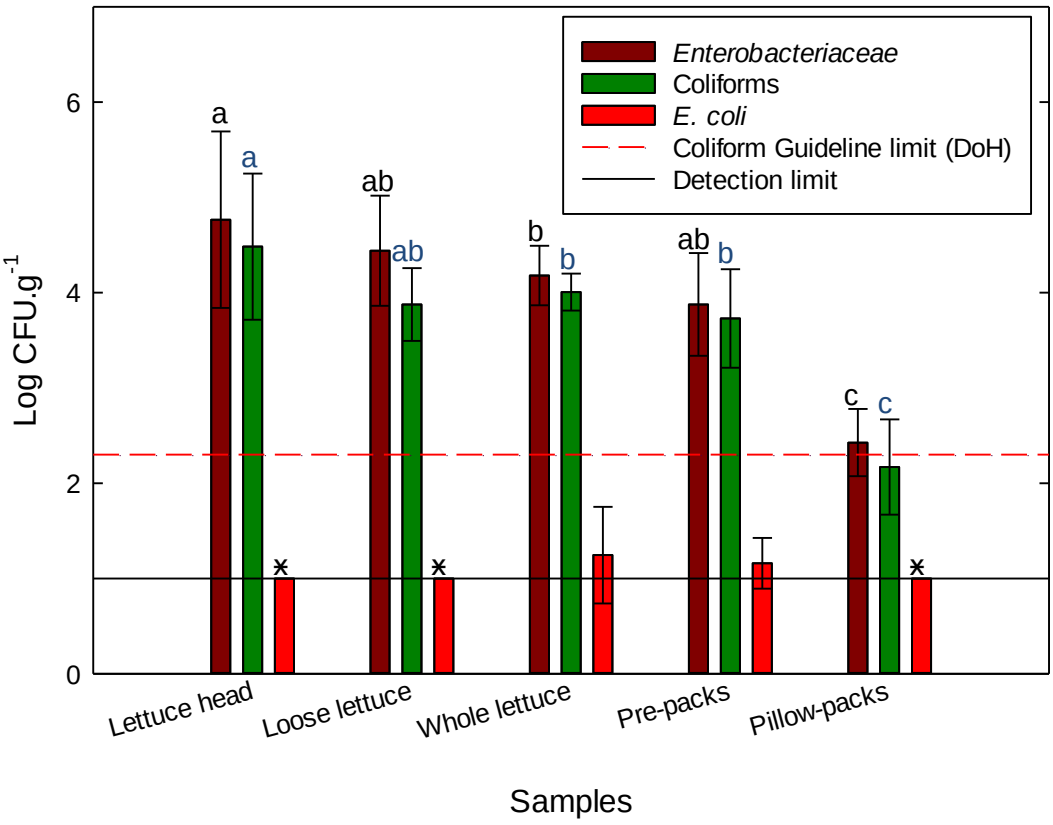

Figure 3. 8 The average levels of *Enterobacteriaceae*, coliforms and *E. coli* on lettuce samples and the significant differences between samples. Bars with the same letters indicate average counts that are not significantly different at 95% confidence level ($p<0.05$). Black and blue letters represent *Enterobacteriaceae* and coliforms respectively. ⁎=not detected. The error bars indicate standard deviation. The red dotted line indicates the highest accepted level of coliforms on ready to eat fresh produce, by the South African department of Health (DoH) (DoH, 2002). The black line indicates the lowest level at which microorganisms could be detected (1 log CFU.g^{-1}).

The processing steps of pre-packaged lettuce are described in Figure 2. There was no significant difference ($p<0.05$) between *Enterobacteriaceae* and coliforms level of pre-packaged and whole lettuce (Fig. 3.8). However, the level of *Enterobacteriaceae* and coliforms average counts on pre-packaged lettuce (3.88 and 3.73 log CFU.g^{-1}, respectively) was significantly ($p<0.05$) lower than *Enterobacteriaceae* and coliforms average counts on unprocessed lettuce head (4.77 and 4.42 log CFU.g^{-1}) (Figure 3.8). The average counts of *Enterobacteriaceae* and coliforms between pre-packaged lettuce and whole lettuce (outer leaves removed, unwashed) were not significantly different ($p= 0.36$; $p= 0.33$ respectively). These results indicate that the washing method was not effective enough to significantly remove microorganisms from whole lettuce. The contact time change in pH level, and the reaction of chlorine with organic matter could be the cause of ineffective disinfection in this study. The ideal contact time observed in literature ranges from 1 to 5 minutes (Goodburn & Wallace, 2013; Ramos *et al.*, 2013), and the pH<8 is used. Studies have indicated that the effectiveness of chlorine disinfection can be lowered by accumulation of organic matter from

fresh produce during washing, which may react with chlorine and form by-products, consequently, inactivating chlorine activity/efficacy (Gil *et al.*, 2019; Stefán *et al.*, 2019). The presence of by-products in wash water reduces the efficiency of chlorine disinfection activity (Gil *et al.*, 2019; Stefán *et al.*, 2019).

Escherichia coli was recovered from whole lettuce (lettuce head with outer leaf removed, unwashed) and pre-packaged samples in low levels (Figure 3.8), below suggested levels (100 CFU.g^{-1}) of the European Commission (EC, 2007). The presence of *E. coli* could be a result of contamination during handling, which could come from packers hands, surfaces or could be samples from produce that were contaminated during transportation from farm to the pack-house (Gil *et al.*, 2015; Rajwar *et al.*, 2016). Pillow-packs samples had the lowest average counts of *Enterobacteriaceae* and coliforms. *E. coli* was not recovered from pillow-packs samples. Pillow-packs samples were prepared from the lettuce head as follows: cutting off the stem, wash loose leaves in chlorine water at a concentration of 150-200 ppm, then spin and package. The result obtained from the pillow-packs samples indicates significantly lower *Enterobacteriaceae* and coliforms average counts (2.42 and 2.15 log CFU.g^{-1}) ($p<0.05$) than the lettuce head average counts. Spinning of lettuce leaves removes excess water which could facilitate the growth of microorganisms (Sela & Fallik, 2009). Therefore, the lower water activity could be the reason why pillow-packs samples had the lowest microbial counts. Another reason could be that pillow-packs were not shredded as this provides growth nutrients to the microorganisms.

CONCLUSIONS

The microbiological quality of broccoli, cabbage, carrot and lettuce sampled from different processing steps within the pack-house was successfully determined. Peeling and washing were the most important processing steps which remove microorganisms from fresh produce. An increase in the level of microorganisms after shredding was considered to be a result of recontamination of fresh produce (from packers hands, surfaces, shredder or packaging material) or exposure of fresh produce to favourable temperatures that support the growth of microorganisms already present on samples. Unwashed samples in this study were found to have high average counts of *Enterobacteriaceae* and coliforms, which were significantly reduced after peeling and washing. The peeling and washing did however not eliminate the organisms from fresh produce, but only reduced them to lower levels. The highest reduction was observed in lettuce pre-packed samples. However, a significant increase in both microorganisms was observed after shredding, and the highest microbial levels (*Enterobacteriaceae* and coliforms) were found in shredded carrot samples. The *E. coli* detected only in some processing steps could either be due to *E. coli* growth from

previously undetectable levels or through post-processing contamination. This is of concern especially on shredded samples because the shreds are mixed to make up a coleslaw mix sold at retail outlets, which are consumed without heat treatment and sometimes with no further washing. Therefore, there is a need for a research study to determine the effective treatment methods of fresh-cut produce that will reduce microbial levels to < 1 log CFU.g^{-1}. This study has also concluded that shredding and packaging could be the potential contamination points, and that higher temperatures could compromise the microbiological quality of fresh produce. A more comprehensive study could be done to also test the processing equipment, surfaces, wash water and worker's hands.

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CHAPTER 4

EVALUATION OF MICROBIOLOGICAL QUALITY OF FRESH PRODUCE PRE- AND POST-PACK-HOUSE PROCESSING, AS WELL AS RETAIL OUTLETS

ABSTRACT

Fresh produce contaminated with microbial pathogens impacts consumers' health. Members of the *Enterobacteriaceae* have been reported to be progressively becoming resistant to many antimicrobial drugs. This makes the use of antimicrobial drugs to treat bacterial infections in patients difficult. Information on the microbial safety, and the potential contamination points of fresh produce supplied to the formal markets in the Western Cape, South Africa is limited. This study aimed at enumerating the microbial indicators: *Enterobacteriaceae*, coliforms and *E. coli,* as well as to test for the presence of microbial pathogens: *Salmonella* and STEC, and the ESBL-producing *Enterobacteriaceae* as well as describing the antimicrobial susceptibility on fresh produce samples collected pre- and post-pack-house processing steps and at the retail outlets. This was done in order to determine the microbial quality of fresh produce along the production chain over-time. A total of 126 samples (18 carrots, 18 broccoli stems, 18 red cabbage, 18 mixed coleslaw bags and 54 lettuce samples) were collected from a pack-house in Phillippi, Western Cape South Africa. *Enterobacteriaceae,* coliforms and *E. coli* overall average levels in produce samples ranged from 1.55 to 6.33 log CFU.g^{-1}, 1.47 to 6.12 log CFU.g^{-1} and <1 to 2.47 log CFU.g^{-1}, respectively in this study. Untreated samples were found with significantly higher microbial levels than treated samples. Samples collected from the retail outlets had significantly higher levels of microorganisms than those sampled from the pack-house. No *Salmonella* or STEC was detected in any of the produce samples. Overall, 89% of the presumptive ESBL-producing organism isolates were identified as *Enterobacter cloacae* (64 %), *Klebsiella oxytoca* (18%) and *E. coli* (7%). Fourteen percent of these isolates were ESBL-producing *Enterobacteriaceae*. Multi-drug resistance was observed in 6% of isolates. Three *K. oxytoca* isolates have co-expressed *bla*$_{TEM}$+$_{CTX-M}$, and the same applies to *E. cloacae* isolates. *bla*$_{TEM}$ was found in *E. coli* isolates. This study highligted the microbial quality of fresh produce sold in South Africa Western Cape Province formal markets.

INTRODUCTION

Fresh produce as part of a healthy diet can also be a transmission vehicle of microbial pathogens to human. Pathogens like *Salmonella* and *E. coli* have been reported globally to be frequently associated with food-borne illnesses linked with fresh produce consumption (Jung *et al.*, 2014; Murray *et al.*, 2018, Centres of Disease Control (CDC), 2019a). *Escherichia coli* exist naturally in the gastro-intestinal tract of humans and animals as a non-pathogenic organisms (Baker *et al.*, 2016). However, some *E. coli* strains produce virulence genes known as shiga-toxins (Stx) which cause infections in humans (Khalil *et al.*, 2015). This type of *E. coli* is referred to as shiga-toxin producing *E. coli* (STEC) (Khalil *et al.*, 2015). STEC infection results into serious human health complications, which include haemorrhagic colitis and haemolytic-uremic syndrome, and deaths are also reported (Singh *et al.*, 2019). Shiga toxin producing *E. coli* (O157:H7) has been the most frequent cause of food-borne outbreaks linked to fresh produce (CDC, 2019b). According to the CDC (2019b), the major STEC responsible for human illness comes from cattle. A study done in the Eastern Cape, South Africa has detected STEC (*E. coli* O157:H7) on fresh produce (Abong *et al.*, 2008).

Salmonella is also a very important pathogen responsible for gastrointestinal illnesses and fever in humans (Fox *et al.*, 2018). Cases of salmonellosis (enteric disease caused by *Salmonella* spp) have been reported in South Africa, linked to consumption of contaminated food (Muvhali *et al.*, 2017). *Salmonella* infection has been commonly associated with consumption of contaminated meat products (Beuchat, 1996; Harris *et al.*, 2003). However, there has been an increase in cases of *Salmonella* linked with contaminated fresh produce consumption (Harris *et al.*, 2003; Jung *et al.*, 2014; Murray *et al.*, 2018). The CDC (2019) has reported recent *Salmonella* multistate outbreaks linked to papaya and pre-cut melons.

Both STEC and *Salmonella* are disseminated in the environment through faecal matter for both animals and humans (Persad & LeJeune, 2018; Dias *et al.*, 2019). The fact that fresh produce is open in nature makes it easier for the organisms to contaminate the produce especially where good agricultural practices, good manufacturing practices and good hygiene practices are not employed (Persad & LeJeune, 2018). Once the produce gets contaminated, it can be difficult to remove the pathogens especially on produce that is eaten raw. Results obtained in the previous Chapter have indicated that washing reduces microorganism number but do not completely remove microorganisms. Therefore, it is crucial to prevent fresh produce contamination from production and throughout the supply chain, to ensure fresh produce safety.

Salmonella and STEC are members of the *Enterobacteriaceae* family. It has been reported that members of the *Enterobacteriaceae* family have been increasingly developing

resistance to the 3rd generation cephalosporins (van Hoek *et al.*, 2015). The third generation cephalosporins are very important antibiotics used to treat infections caused by bacteria. *Escherichia coli* and *Klebsiella* pneumonia were the first organisms to be diagnosed with resistance, however many species are now becoming resistant to antibiotics (Pitout & Laupland, 2008). The organisms develop resistance by producing the extended spectrum β-lactamases (ESBLs) which hydrolyse ampicillin and the 3rd generation cephalosporins (Blaak *et al.*, 2014). Resistance in *Enterobacteriaceae* occurs as a result of ESBLs class A enzymes: bla_{TEM}, bla_{SHV} and bla_{CTX-M} (Monstein *et al.*, 2007; Richter *et al.*, 2019). Bacteria carrying resistant genes are found in animals' faeces and humans stools (van Hoek *et al.*, 2015). Through animal manure used as compost, and faecal contaminated irrigation water or surfaces, the ESBL-producing organisms end up on fresh produce (van Hoek *et al.*, 2015). With increasing consumption of raw fresh produce, the transmission of ESBL-producing organism to humans could be high. This might be a risk to humans' health. ESBL-producing bacteria express resistance to many antimicrobial drugs, limiting the use of available antimicrobial agents. Ineffective treatment with third generation cephalosporins against some infections has been reported in South Africa (World Health Organization (WHO), 2019). ESBL-producing bacteria have been found on fresh produce sampled from formal and informal retailer in Gauteng Province, South Africa (Richter *et al.*, 2019), and also from fresh produce in other countries (van Hoek *et al.*, 2015; Zurfluh *et al.*, 2015). In the Western Cape, the ESBL-producing bacteria were detected on fresh produce collected from the informal markets (Laubscher, 2019). However, there is still a gap regarding formal markets fresh produce in the Western Cape.

This study aims to enumerate microbial indicators, and to test for the presence of Shiga toxin producing *E. coli* (STEC), *Salmonella*, and the ESBL-producing *Enterobacteriaceae* on produce collected before and after pack-house processing steps, and at the retail outlet, in order to determine the changes in microbiological quality of fresh produce along the production chain (pack-house to retail outlets) overtime.

MATERIALS AND METHODS

Site and sample selection

Samples were collected from the same pack-house in Phillipi, (Western Cape South Africa) where the samples tested in Chapter 3 were collected from. Lettuce samples (lettuce head, loose lettuce, Pre-packs and pillow-packs) (see definitions in Table 4.2), and broccoli coleslaw samples (carrots, broccoli stem, cabbage, and mixed bags) (see definitions in Table 4.1) were collected on the day of processing at the pack-house. Samples (coleslaw

bags, and Pre-packs) from the same batch as samples collected the day of processing were also bought two days after processing from the retail outlets where the fresh produce from the pack-house was sold. This was done to determine the changes in the microbiological quality of produce of the same batch collected from both the pack-house right after processing and retailers two days after processing.

Sampling design

A total of 18 broccoli stem, 18 carrots, 18 red cabbage, 18 mixed coleslaw bags, and 54 lettuce samples were collected in this study. Different sample types were collected as indicated in Figures 4.1 (broccoli coleslaw) and 4.2 (lettuce samples). In this Chapter, packaged samples from the same batch were collected at both the pack-house as well from the retail outlet two days later where it was sold to the public except for the lettuce pillow-packs.

All samples were collected in triplicate during a sampling event. Sampling events from the pack-house as well as retail outlets were also repeated three times for each produce type. Upon sampling, samples were packed in sealable plastic bags and put in a cooler box with crushed ice, after which it was transported to the Food Science Department. Upon arrival, samples were stored at 4^{o}C until analysis, which was done within 24 hours.

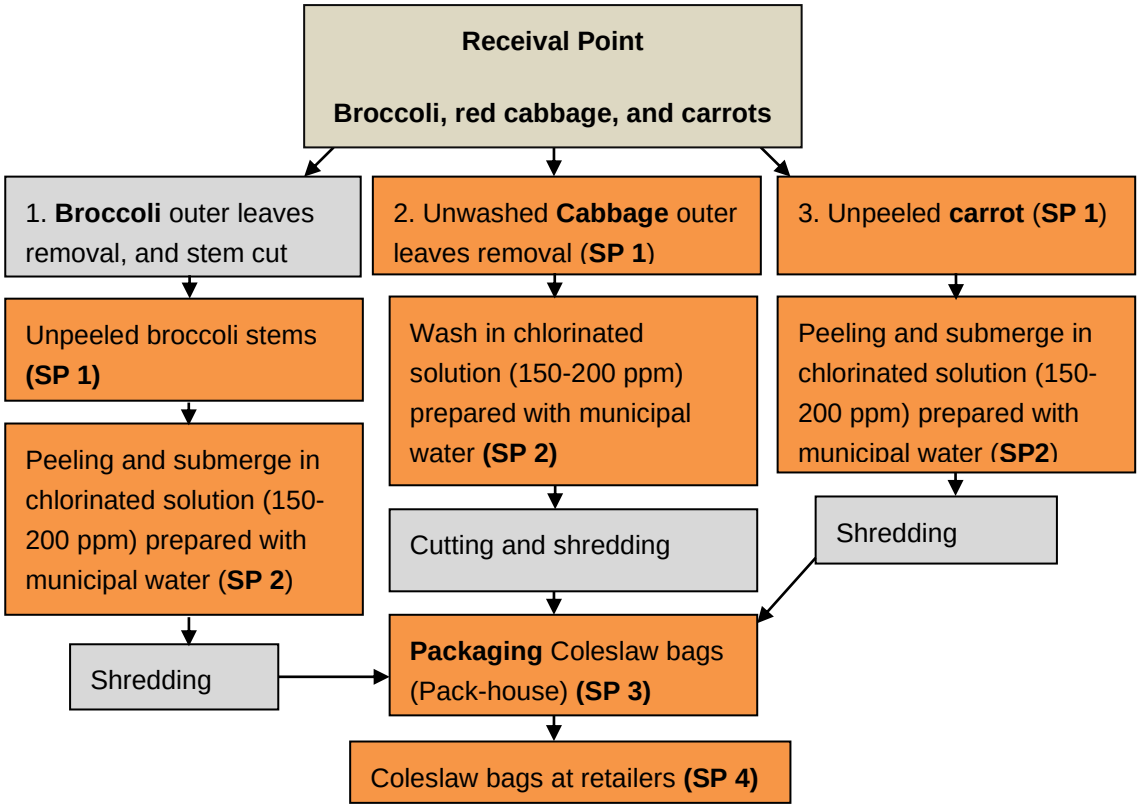

Figure 4.1 Broccoli coleslaw processing steps and sampling points (SP)

Table 4. 1 Description of terms in the processing steps and sampling points in Figure 4.1

Terms	SP	Definitions
Untreated broccoli stem	SP1	Broccoli stem that is not peeled or washed.
Treated broccoli stem	SP2	Broccoli stem that is peeled and submerged in chlorine solution (150-200 ppm) prepared with municipal water, for at least a minute.
Untreated cabbage	SP1	Unwashed cabbage with outer leaves removed.
Treated cabbage	SP2	Cabbage with outer leaves removed washed in chlorine solution (150-200 ppm) prepared with municipal water, for at least a minute.
Untreated carrot	SP1	Carrot that is not washed or peeled.
Treated carrots	SP2	Peeled carrots submerged in chlorine solution (150-200 ppm) prepared with municipal water, for at least a minute.

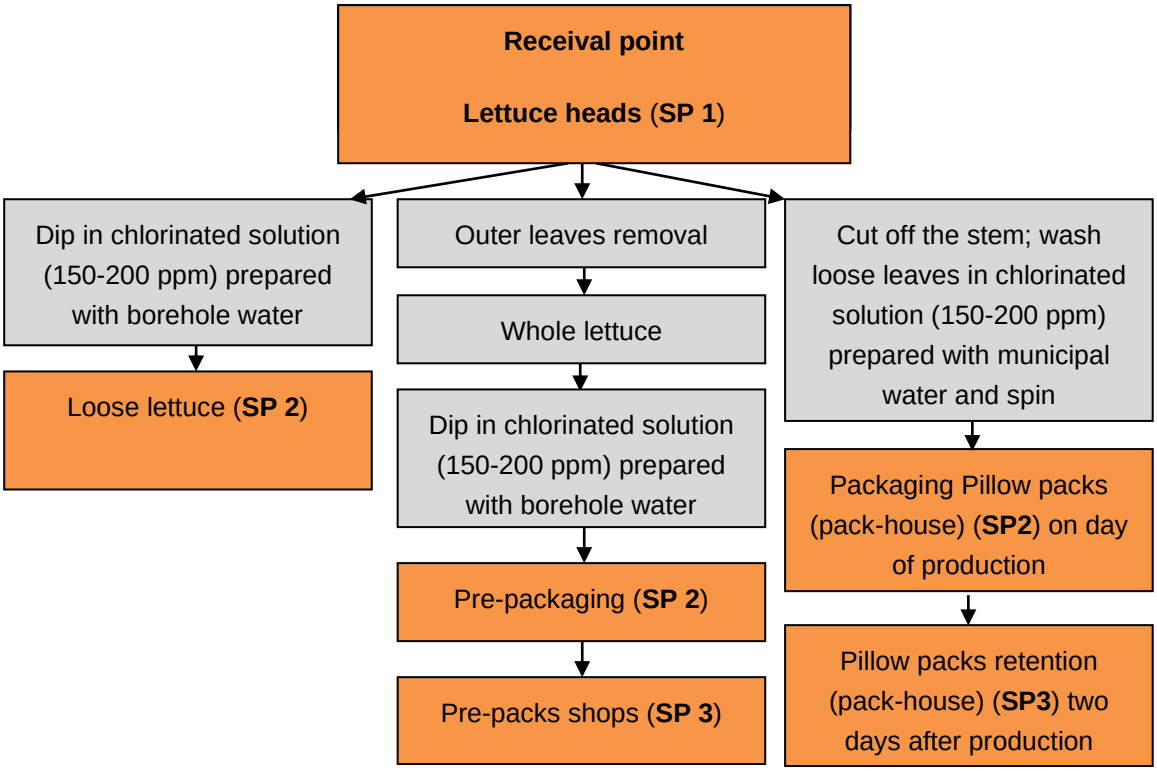

Figure 4.2 Lettuce samples processing steps and sampling points (SP)

Table 4. 2 Description of terms in the processing and sampling points in Figure 4.2

Terms	SP	Definition
Lettuce head	SP 1	Unprocessed lettuce
Loose Lettuce	SP 2	Loose lettuce head dipped in chlorine solution (150-200 ppm) prepared with borehole water, for at least a minute
Pre-packs	SP 3	Whole lettuce dipped in chlorine solution (150-200 ppm) prepared with borehole water, for at least a minute; packaged and labelled as wash before use.
Pillow-packs	SP 2&3	Ready-to-eat lettuce leaves.

MICROBIOLOGICAL ANALYSIS

Sample preparation

To test for microbial indicators (*Enterobacteriaceae*, coliforms, *E. coli*), *Salmonella*, STEC and ESBL-producing *Enterobacteriaceae*, all samples were prepared as described in Chapter 3. In short, from each sampling point (SP) indicated in Figures 4.1 and 4.2, sampling was done in triplicate. Triplicate samples were prepared by cutting each sample in half, and then the half of each of these samples was cut into smaller pieces on a sterile metal tray, with a sterile knife. A 100 g was then collected from each tray and mixed on a separate sterile tray to obtain a 300 g composite sample. From the composite sample, three triplicate 25 g samples were collected and transferred into three separate sterile polyethylene stomacher bags. Thereafter, 225 mL of sterile 0.1% buffered peptone water (BPW) (Merck, South Africa) was added to the bags containing 25 g samples and stomached at 230 rpm for 2 min in a 220V Interscience Bag Mixer (SANS, 6887-3:2004).

General materials and methods

To determine the microbial changes on fresh produce along the supply chain from pack-house to retailer, microbial indicators (*Enterobacteriaceae*, coliforms, and *E. coli*) were enumerated as in Chapter 3. This study also included testing for two pathogens: STEC and *Salmonella*, as well as detection and isolation of ESBL-producing *Enterobacteriaceae.* Buffered peptone water (BPW) (Merck, South Africa) was used as a primary enrichment media. The detection of STEC and *Salmonella* was done using DuPont™ BAX® system. However, the manufacturer recommends a BAX® system MP enrichment media as a primary enrichment media for samples to be tested with the BAX® system. In the absence of the BAX® system MP® enrichment media, BPW (Merck, South Africa) was used as an alternative primary enrichment media. Therefore, to ensure reliable results, it was necessary to determine the compatibility of BPW with the BAX® system, and to determine the sensitivity of the BAX system in detecting both *Salmonella* and STEC (*stx* and *eae*) before testing the study samples.

Validation methods for pathogen detection

To determine the compatibility of BPW as a primary enrichment media with the BAX® system, STEC and *Salmonella* detection was done using BAX Real Time PCR Assay STEC screening *stx & eae* kit (Microsep), and BAX PCR Assay for screening *Salmonella* 2 kit (Microsep) respectively. Validation was done using STEC and *Salmonella* reference strains,

which were prepared, enumerated and spiked on fresh cabbage cuts before BAX® system testing.

Inoculums preparation

Salmonella ATCC 14028 and *E. coli* (STEC) 210 strains previously isolated from game meat (P. Gouws, 2018, Department of Food Science Stellenbosch University, personal communication) were used for the BAX® system validation process. Strains were prepared from stock cultures by inoculating 200 µL of each strain individually into 5 mL of sterile Tryptone Soy Broth (TSB) (Oxoid, South Africa) and incubated at 37°C for ± 24 h. To confirm the purity of strains, a loop full of *E. coli* suspension was streaked on Levine's Eosine Methylene-Blue Agar (LEMB) (Oxoid, South Africa), and a loop full of *Salmonella* suspension was streaked on two selective differential growth media: Xylose Lysine Deoxycholate (XLD) agar (Oxoid, South Africa), and Hektone agar (Oxoid, South Africa). *Escherichia coli* growth was identified as metallic green sheen colonies on L-EMB agar. *Salmonella* growth on XLD appeared as red colonies with a black centre on XLD agar, and as blue green colonies with a black centre on Hektone agar. For each strain, a single colony was picked and inoculated in 5 mL TSB (Oxoid, South Africa) and incubated at 37°C for 24 h. Following incubation, 500 µL of each strain was transferred individually to 1.5 mL centrifuge tubes, and centrifuged (Neofuge 13, Vacutec) for 5 minutes at 14 000 × g. The supernatant was then discarded, and the pellet was rinsed in 500 µL sterile Ringers solution and centrifuged once more for 2 minutes at 14 000 × g, and then re-suspended in sterile Ringers solution to a final turbidity comparable to 0.5 McFarland standard (BioMèrieux, South Africa) with an estimated microorganism concentration of 10^{-7} CFU.mL^{-1}. The absorbance was read at 600 nm and an OD value of 0.27 for *Salmonella* and 0.16 for *E. coli* was obtained.

Spiking with positive strains

Red cabbage sourced from the pack-house (and previously washed in chlorine (150-200 ppm) solution), was re-washed with sterile distilled water to remove possible contaminants. It was then cut into smaller pieces on a sterile tray with a sterile knife and weighed into eight sterile bags (stomacher bags) of 25 g each. The freshly prepared inoculums (*Salmonella* and *E. coli*) with estimated concentration of 10^{-7} CFU.mL^{-1} were individually serially diluted in the range of 10^{-2} to 10^{-7}, by transferring 1 mL of each suspension into 9 mL of Ringers solution. Individually, 500 µL of the highest three dilutions 10^{-7}, 10^{-6} and 10^{-5} (cell concentrations estimated to be in log range of 10^{0}-10^{1}, 10^{1}-10^{2}, and 10^{2}-10^{3}) were spiked on the previously weighed (25 g) red cabbage samples. This was done in order to determine the BAX® system detection sensitivity, and compatibility with BPW as a primary enrichment media is. The

spiking doses were also plated out in duplicate on VRBG agar to confirm the concentration of each spiking dose. Two bags of 25 g red cabbage samples were not spiked and used as negative controls. Spiked samples were left to dry for about 10 minutes, after which BPW (225 mL) was added, and bags were stomached as previously explained in Chapter 3. The stomached samples were then incubated at 37ºC for 24 h.

Pathogen detection

Following incubation, the presence or absence of *Salmonella* and STEC was confirmed using the DuPont™ BAX® system according to the instruction of the manufacturer: 20 µL of STEC, and 5 µL of *Salmonella* enriched samples were individually transferred to prepared 200 µL BAX lysis reagents in cluster tubes. Lysis was followed by heating the tubes at 37ºC for 20 min and denaturing at 95ºC for 10 min. The tubes were then cooled at 4ºC for 5 min. Thereafter, 30 µL and 50 µL of STEC and *Salmonella* lysate, respectively, were transferred to the PCR tubes to hydrate the tablet in the tube. The tubes were then sealed and loaded into the BAX® System Q7 instrument, and a full process was run, and analysed by the software version 3.2 standard assays. The run for STEC and *Salmonella* were analysed separately.

STEC isolation

STEC isolation was done according to Kim *et al.* (2014) with some modifications. The 25 g produce samples that were homogenised in 225 mL BPW, were incubated at 35ºC for 24 h. Following incubation, 1 mL cultured sample was transferred to 9 mL of *Escherichia coli* (EC) broth (Oxoid, South Africa) then incubated at 35ºC for 24 h. After incubation, the EC broth with sample was streaked onto L-EMB agar (Oxoid, South Africa), and incubated at 35ºC for 24 h. A single *E. coli* colony was transferred to 5 mL of TSB and incubated at 37ºC overnight. After incubation, 800 µL was stored at -80ºC in 40% sterile glycerol. For confirmation, the BAX® system was used to test STEC on isolated colonies.

Salmonella spp isolation

Isolation of *Salmonella* was done according to the SANS 6579:2003, and the Bacteriological Analytical Manual (BAM) (Andrews, *et al*., 2011). Samples (25 g) homogenised in 225 BPW and incubated at 35ºC for 24 h. After incubation, 0.1 mL was transferred to 10 mL Rappaport-Vassiliadis Soya (RVS) broth (Oxoid, South Africa), and then incubated at 42ºC for 24 h. A loop full was then streaked on two selective differential media: XLD and Hektone agar and incubated at 35ºC overnight. After incubation, single colonies were picked and streaked on nutrient agar for purity, and then incubated at 37ºC for 24 h. Presumptive

Salmonella growth was identified by black colonies on XLD agar and dark-green colonies on Hektone agar. A pure colony was then picked from nutrient agar and transferred into 5 mL TSB and incubated at 37ºC for 24 h. After incubation 800 µL was stored at -80ºC in 40% sterile glycerol. The conserved cultures were tested again to confirm *Salmonella* with the BAX® system.

Experimental Study

Enumeration of Enterobacteriaceae, coliforms, and E. coli

Enumeration of *E. coli* and *Enterobacteriaceae* was done as previously explained in Chapter 3. In brief, serial dilutions (10^{-2}– 10^{-5}) were prepared by transferring 1 mL of the homogenised sample to 9 mL of Ringers solution according to the SANS 6887-3:2004 method. This was followed by plating out each dilution in duplicate, on Rapid *E. coli* 2 agar (Bio-Rad, South Africa) for coliforms and *E. coli* following the SANS 4832:2006 method, and onto Violet Red Bile Glucose Agar (VRBGA) (Oxoid, South Africa) to enumerate *Enterobacteriaceae,* according to the SANS 21528-2:2005 method. Standard aseptic pour plate method was used. All plates were then incubated at 37±2ºC for 24 h, after which colonies types and numbers were recorded. Counting was done on plates that had 10-300 colonies.

Pathogen detection and isolation

Detection and isolation of Salmonella and STEC

This study aimed at detection and isolation of *Salmonella*, STEC and ESBL-producing *Enterobacteriaceae*. Following sample preparation, the same bag from where dilutions were prepared, was incubated at 37ºC for 24 h. Samples were then tested for *Salmonella* and STEC using the DuPont™ BAX® system as described in validation methods. The Bax real-time PCR assay kit for screening STEC (*stx* and *eae*) genes (Microsep) and Bax PCR assay kit for screening *Salmonella* 2 (Microsep) were used. The positive samples detected by the BAX® system were isolated and conserved as described previously in the validation methods.

ESBL-producing Enterobacteriaceae detection and Isolation

Detection of ESBL producers was done according to the procedure of Zurfluh *et al.* (2015), with a few modifications to suit this study. Samples (25 g) homogenised in 225 BPW were incubated at 37ºC for 2 h. After incubation, 1mL was transferred into 10 mL of *Enterobacteriaceae* enrichment (EE) broth (Merck, South Africa), and then incubated at

37ºC for 24h. A loop full was then streaked on ChromID Brilliance ESBL agar (bioMèrieux, South Africa) and incubated at 37ºC for 24 h. The ChromID screening agar contains cefpodoxime, two chromogenic substrates, and a natural substrate, which allows for direct species identification. Any growth on plates was considered as presumptive ESBL positive colonies. Colonies colours were recorded according to the manufacturer' colour chart (Figure 4.3). The presumptive colonies were then sub-cultured into TSB (Merck, South Africa), and 800 µL was stored in 40% glycerol at -80ºC until further analysis.

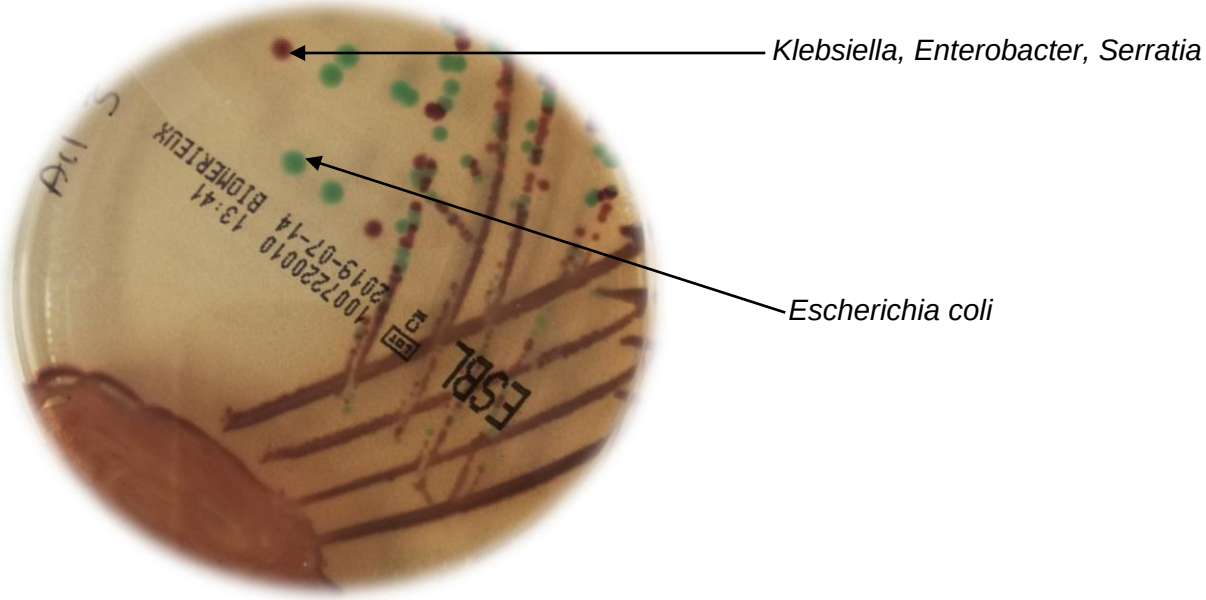

Figure 4.3 Different colonies growth on chromID agar indicating the presence of different organisms

Identification of ESBL-producing isolates

Different coloured colonies that were isolated from chromID Brilliance ESBL plates were considered to be presumptive ESBL producers. Strain identification was done using Matrix-Assisted Laser Desorption/Ionisation-Time of Flight (MALDI-TOF) mass spectrometry (Bruker Daltonics GmbH, Germany). This was done by transferring one 1 µL of each prepared isolate supernatant onto a multi-samples stainless steel loading plate (Bruker Daltonics GmbH, Germany). The spotted samples were allowed to dry at room temperature. Each sample was then covered with 1 µL of alpha-cyano-4-hydroxycinnamic acid matrix saturated solution (Bruker Daltonics GmbH, Germany) and left to dry at room temperature. Two spots for each isolate were prepared systematically, after which isolates were subjected to MALDI-TOF analysis. The results obtained with MALDI-TOF Biotyper (Bruker Daltonics GmbH, Germany) given by the Bruker Daltonics MALDI-TOF Biotyper were classified by colour-coded symbols as follows: (+++) green = highly probable species identification, (++)

green = secure genus identification, probable species identification, (+) yellow = probable genus identification, (-) red = not reliable identification.

Antimicrobial susceptibility test confirmation of ESBL-producing Enterobacteriaceae

Antimicrobial susceptibility testing was done on all fresh produce isolates identified as *Enterobacteriaceae* with MALDI-TOF. This was done by following the European Committee on Antimicrobial Susceptibility Testing procedures (EUCAST, 2017a). All the isolates were streaked onto nutrient agar (Oxoid, South Africa) and incubated. A colony of each isolate was then suspended in sterile distilled water to a final turbidity equivalent to the 0.5 McFarland standard (bioMèrieux, South Africa). The suspension was then used to inoculate on Mueller-Hinton (MH) agar plates in duplicate (Davis Diagnostics, South Africa) with a sterile cotton swab. Within 15 minutes after plate inoculation, the antimicrobial discs were applied with a dispenser (Davis Diagnostics, South Africa). Plates were incubated within 15 minutes after discs application at 36±1^{o}C for 20 h. The following discs were used to confirm ESBL-producing *Enterobacteriaceae* isolates: ceftazidime (CAZ 30 µg), cefotaxime (CTX 30 µg), and cefepime (CPM 30 µg) (Davis Diagnostics, South Africa). All discs were used with and without (+/-) clavulanic acid (CA 10 µg) (Davis Diagnostics, South Africa). CAZ 30 µg and CTX 30 µg with and without CA (10 µg) were used to confirm group 1 ESBL producers. The results were recorded as positive for group1 when the growth inhibitory zone diameter around CAZ+CA (30+10 µg) or CTX+CA (30+10 µg) was ≥5 mm larger than the inhibitory zone diameter around CAZ (30 µg) or CTX (30 µg) disc without CA (10 µg) (EUCAST, 2017b). Group 2 ESBL producer were confirmed as positive when the growth inhibitory zone around CPM+CA (30 µg+10 µg) was ≥5mm larger than CPM (30 µg) without CA (10 µg) (EUCAST, 2017b). Fresh produce isolates were also tested for susceptibility to five other antibiotics from different classes (Table 4.3). The resistance of fresh produce isolates to the five chosen antibiotics has previously been reported (Zurfluh *et al.*, 2015; Du Plessis *et al.*, 2017).

Table 4. 3 Six additional antibiotics used for susceptibility testing in this study

Classes	Antibiotics	Concentration (µg)
Tetracycline	Tetracycline	30
Ampenicols	Chloramphenicol	30
Fluoroquinolone	Ciprofloxacin	5
Aminoglycoside	Gentamicine	10
Penicillin	Ampicillin	10

A ruler was used to measure the diameter of the inhibition zones. All susceptibility testing was done in duplicate. The results were used to classify strains as sensitive, intermediate, or resistant according to the Clinical and Laboratory Standards Institute (CLSI, 2016) interpretive criteria for *Enterobacteriaceae* (Table 4.4). To confirm method validity, two reference strains: (1) *Escherichia coli* ATCC 25922 which is susceptible to most antibiotics, and (2) *Escherichia coli* ATCC 35218 (which is resistant to amplicillin and other antibiotics due the presence TEM-1 β-lactamase) (EUCAST, 2017a) were used in this study as negative and positive controls respectively.

Table 4.4 Criterion for interpreting the inhibition zone diameter of antibiotics resistance of *Enterobacteriaceae* (CLSI, 2016)

Antibiotics	**Inhibition zones diameter**		
	Sensitive ≥ (mm)	Intermediate (mm)	Resistant ≤ (mm)
Amplicillin 10 µg	17	14 – 16	13
Chloramphenicol 30 µg	18	13 – 17	12
Tetracycline 30 µg	15	12 – 14	11
Gentamicine 10 µg	15	13 – 14	12
Ciprofloxacin 5 µg	31	21 – 30	20

Genotypic confirmation (ESBL genes detection)

To confirm the presence of ESBL genes (*bla*-$_{SHV}$, *bla*-$_{TEM}$, and *bla*$_{CTX-M}$) on isolates, a multiplex polymerase chain reaction (PCR) was used according to the method described by Monstein *et al.* (2007). This confirmation was performed on all isolates that were identified by MALDI-TOF as *Enterobacteriaceae* in this study. The PCR primer pairs sequences presented in Table 4.5 were used for multiplex PCR in this study. The cell extracts used for PCR was prepared from isolates that were identified as *Enterobacteriaceae* by the MALDI-TOF.

Preparation of cell extracts

The cell extracts used as PCR templates were prepared according to the boiling method of Altalhi & Hassan (2009). It was done as follows: the bacterial cultures were grown on nutrient agar (Merck, South Africa) overnight at 37^{o}C. A single colony was isolated and suspended into 100 µL sterile nuclease free water (BioConcept, Switzerland). The

suspension was boiled for 13 minutes and centrifuged at 14000 × g for 15 min to separate the cell debris from the supernatant. The supernatant was transferred to sterile 0.6 mL PCR tubes and stored at -20ºC until PCR analysis.

Multiplex polymerase chain reaction (PCR) and electrophoresis

All PCR reactions were carried out in 25 µL volumes which consisted of 12.5 µL One Taq Quick-Load Master Mix with Standard Buffer (Biolabs, New England), 3 µL primer mix (BioLabs, New England), 1 µL DNA template, and 8.5 µL of sterile RNase-free water. Each 25 µL PCR reactions were transferred to the thermocycler (Vacutec, South Africa), and amplification was achieved through the following conditions: initial denaturation at 95ºC for 15 min, followed by 30 cycles of denaturation at 94ºC for 30 s, annealing at 60ºC for 30 s, and extension for 2 min at 72ºC. The final extension was at 72ºC for 10 min, after which samples were held at 4ºC. The PCR products were visualised by gel electrophoresis. The gel electrophoresis was performed in 1.2% agarose gel (SeanKem, Switzerland) containing 1 µL.10 g^{-1} EZ-Vision blue-light DNA dye (VWR Life Science, USA), in 0.5% TAE buffer. Bands were visualised with the UV-transilluminator (Vacutec, South Africa). Sterile RNase water (1 µL) was used as a negative control. *Escherichia coli* (ATCC 35218) containing $bla_{TEM,}$ *Klebsiella pneumonia* (ATCC 700603) containing bla_{SHV}, and *Klebsiella pneumonia* containing containing bla_{CTX-M} were included in PCR as positive controls. A 100-base pair (bp) DNA ladder (BioLabs, New England) was included as a band size marker during electrophoresis. Bands were identified through visual comparison with the bands of the positive controls as well as the base pair marker. The expected band lengths of the targeted genes are presented in table 4.5.

Table 4. 5 The primer pairs used for amplification of ESBL genes (SHV, TEM, and CTX-M)

Target	Primer sequence	Size (bp)	Reference
Bla-SHV.SE	5'-ATGCGTTATATTCGCCTGTG-3'	747	Paterson *et al*., 2003
Bla-SHV.AS	5'-TGCTTTGTTATTCGGGCCAA-3'		
TEM-164.SE	5'TCGCCGCATACACTATTCTCAGAATGA-3'	445	Monstein *et al*., 2007
TEM-165.AS	5'ACGCTCACCGGCTCCAGATTTAT-3'		
CTX-M-U1	5'ATGTGCAGYACCAGTAARGTKATGGC-3'	593	Boyd *et.al*., 2004
CTX-M-U2	5'TGGGTRAARTARGTSACCAGAAYCAGCGG-3'		

STATISTICAL ANALYSIS

Colony counts were converted to log values (log CFU.g^{-1}) prior to statistical analysis. Sigma Plot version 13 software was used to calculate and plot the mean values and standard deviations. One-way ANOVA's were conducted to compare average measurements between treatments. For post hoc testing, Fisher Least Significant Difference testing was done. Homogeneity of variance was tested using Levene's test, and for the cases where this hypothesis was rejected, the Welsh test was done with Games-Howell post hoc testing. The P-value ($P<0.05$) was used to determine the statistical significance, at 95% confidence interval (Prof. Martin Kidd, 2019. Centre of Statistical Analysis, Department of statistic, Stellenbosch University, personal communication, 27 September)

RESULTS AND DISCUSSION

Validation study

Detection of STEC and *Salmonella* was done using the BAX® System. A validation study was performed to test the sensitivity of the BAX® system, and also to test whether BPW (which was used as a primary enrichment media instead of the recommended BAX® system MP® enrichment media), would be compatible with BAX® System. Positive controls (STEC and *Salmonella*) spiking doses (Table 4.6) were plated on VRBG agar (Oxoid, South Africa) since both strains are gram negative, part of the *Enterobacteriaceae* family and can both grow well on VRBG agar. Colony counts were recorded (Table 1) and used to confirm the spiking dose used.

Table 4. 6 Colony counts detected at the spiking dose used on red cabbage samples for Salmonella and STEC detection.

	Estimated spiking dose range		
	10^0-10^1	10^1-10^2	10^2-10^3
Actual CFU.mL^{-1} for control strains used for spiking			
Salmonella	2 CFU.mL^{-1}	25 CFU.mL^{-1}	190 CFU.mL^{-1}
Shiga toxin producing *E. coli* (STEC)	3 CFU.mL^{-1}	42 CFU.mL^{-1}	280 CFU.mL^{-1}

The colony counts obtained (Table 4.6) indicated that the spiking doses used were accurately calculated. The counts obtained were corresponding with the spiking dose range.

The analysis results obtained from the BAX® System indicated the compatibility of BPW with the BAX® System. Results (Table 4.7) also indicated that *Salmonella* and STEC

can be detected with the BAX® System even when present in small quantities. *Salmonella* was detected at a concentration at 10^1-10^2 CFU.g^{-1}, but not at very low concentrations (10^0-10^1 CFU.g^{-1}). STEC was detected at 10^0-10^1 CFU.g^{-1}

Table 4. 7 Results at different spiking dose obtained from the BAX System. Positive detection is represented by a plus sign (+) and negative detection by a negative sign (-)

	Spiking dose ranges (CFU. mL^{-1})			
Positive Controls	10^0-10^1	10^1-10^2	10^2-10^3	Negative Contol
Salmonella	-	+	+	-
STEC	+	+	+	-

Experimental study

Prevalence of microbial indicators (Enterobacteriaceae, coliforms, and E. coli) on untreated and treated broccoli stems samples

Enterobacteriaceae, coliforms and *E. coli* were respectively detected on 100%, 89%, and 50% of broccoli samples tested. As previously observed in Chapter 3, the untreated broccoli samples had significantly ($P<0.05$) higher microbial counts before washing and peeling. After peeling and washing the broccoli stems in chlorine solution (150-200 ppm), the average levels of *Enterobacteriaceae* (3.41 log CFU.g^{-1}) and coliforms (3.3 log CFU.g^{-1}) that were detected on untreated broccoli stems was significantly ($P<0.05$) reduced to 2.38, and 2.02 log CFU.g^{-1} (Figure 4.4). Coliform levels were reduced to levels within the DoH guidelines limits (< 200 CFU.g^{-1}) (DoH, 2002). *Escherichia coli* were also recovered from both untreated and treated broccoli samples. However, there was no significant difference ($P=0.19$) in *E. coli* levels recovered from untreated (1.7 log CFU.g^{-1}) and treated (1.3 log CFU.g^{-1}) broccoli stem. In this pack-house treated broccoli stems are shredded to be used in coleslaw bags. The microorganisms left after washing could proliferate in the coleslaw bags. Therefore, the presence of *E. coli* on treated samples is a major concern. The previous guideline limit (under review) set by DoH suggests no *E. coli* on fresh produce that is intended to be eaten raw (DoH, 2002). However, the European Commission (EC, 2007) guidelines suggests ≤ 2 log CFU.g^{-1} as the satisfactory level of *E. coli* on ready-to-eat fresh produce. Therefore, the *E. coli* results are within the European commission's guidelines.

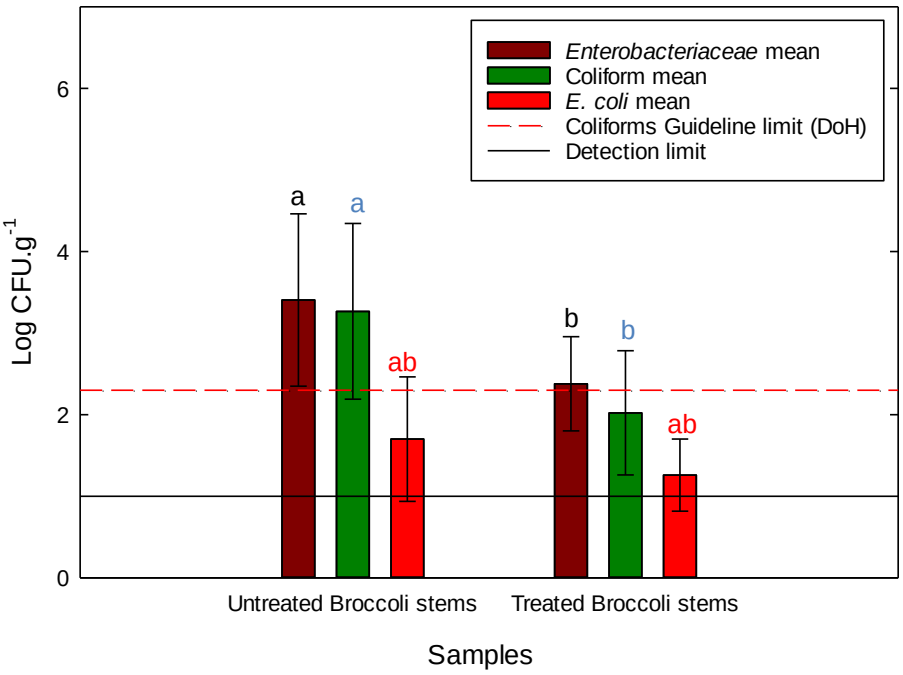

Figure 4.4 Average levels of *Enterobacteriaceae*, coliforms, and *E. coli* on untreated and treated [peeled & washed in chlorine solution (150-200 ppm)] broccoli stems samples, and the significant differences. Bars with different letters indicate average counts that are significantly different at a 95% confidence level (P<0.05). Bars with the same letters indicate average counts that are not significantly different (P>0.05). Black letters represent *Enterobacteriaceae*; blue letters represent coliforms. ⁎=not detected. The error bars indicate standard deviation. The red dotted line indicates the highest accepted level of coliforms on ready-to-eat fresh produce, according to the South African Department of Health (DoH) (DoH, 2002). The black line indicates the lowest level at which microorganisms could be detected in this study (1 log CFU^{-1}).

Prevalence of microbial indicators (Enterobacteriaceae, coliform, and E. coli) on untreated and treated carrot samples

Enterobacteriaceae, coliforms and *E. coli* were, respectively, detected on 83%, 83%, and 61% of carrot samples (Fig. 4.5). The levels of *Enterobacteriaceae* (5.57 log $CFU.g^{-1}$), coliforms (5.51 log $CFU.g^{-1}$) and *E. coli* (2.99 log $CFU.g^{-1}$) on untreated carrots were significantly (P<0.05) higher than the levels of *Enterobacteriaceae* (2.55 log $CFU.g^{-1}$), coliforms (2.41 log $CFU.g^{-1}$), and *E. coli* (1.1 log $CFU.g^{-1}$) on treated (peeled and washed in chlorine (150-200 ppm) solution) samples.

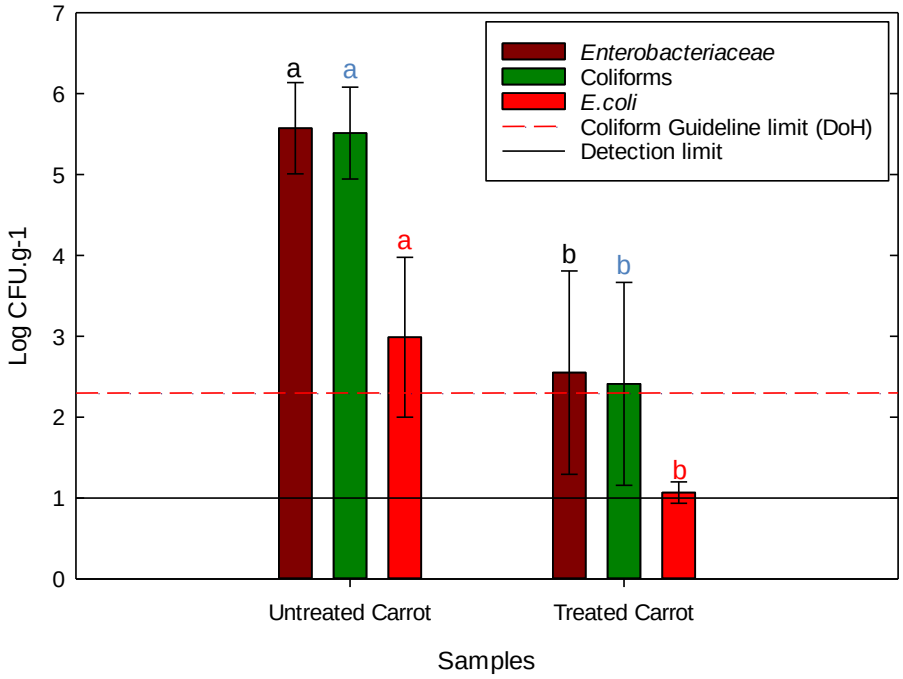

Figure 4.5 Average levels of *Enterobacteriaceae*, coliforms, and *E. coli* on untreated and treated (peeling & chlorine (150-200 ppm) wash) carrot samples, and the significant differences. Bars with different letters indicate average counts that are significantly different at 95% confidence level (P<0.05). Bars with the same letters indicate average counts that are not significantly different (P>0.05). Black letters represent *Enterobacteriaceae*; blue letters represent coliforms. ⁎=not detected. The error bars indicate standard deviation. The red dotted line indicates the highest accepted level of Coliforms on ready-to-eat fresh produce, by the South African Department of Health (DoH) (DoH, 2002). The black line indicates the lowest level at which microorganisms could be detected in this study (1 log CFU^{-1}).

Though the reductions of all microbial population on the carrot samples were significant, coliforms average levels (2.41 log $CFU.g^{-1}$) on treated samples were still higher than the previous DoH guideline (under review) (2.3 $CFU.g^{-1}$) for coliforms on ready-to-eat fresh produce (DoH, 2002). The presence of *Escherichia coli* on fresh produce is limited to zero detection by the DoH (under review), however, it is still under the satisfactory levels (2 log $CFU.g^{-1}$) set by the European Commission (EC) (2007). There is no guideline limiting *Enterobacteriaceae* levels on fresh produce.

Prevalence of microbial indicators (Enterobacteriaceae, coliform, and E. coli) on untreated and treated cabbage samples

Enterobacteriaceae, coliforms and *E. coli* were detected on 78%, 78%, and 17% cabbage samples, respectively. Before washing the red cabbage, the initial average levels of *Enterobacteriaceae*, coliforms, and *E. coli* was 3.41, 3.05, and 1.74 log $CFU.g^{-1}$, respectively (Fig. 4.6). After washing the red cabbage in chlorine solution (150-200 ppm), the average levels of *Enterobacteriaceae* and coliforms were significantly (P<0.05) reduced to 1.55 $CFU.g^{-1}$ and 1.47 $CFU.g^{-1}$ respectively. *Escherichia coli* were not recovered from treated

cabbage samples. The chlorine solution has effectively reduced *E. coli* to levels below the detection level (<1 log CFU.g^{-1}). The levels of all tested microorganisms on treated cabbage samples were within the previous DoH guideline limits (DoH, 2002) (under review), as well as the EC (EC, 2007) criterions.

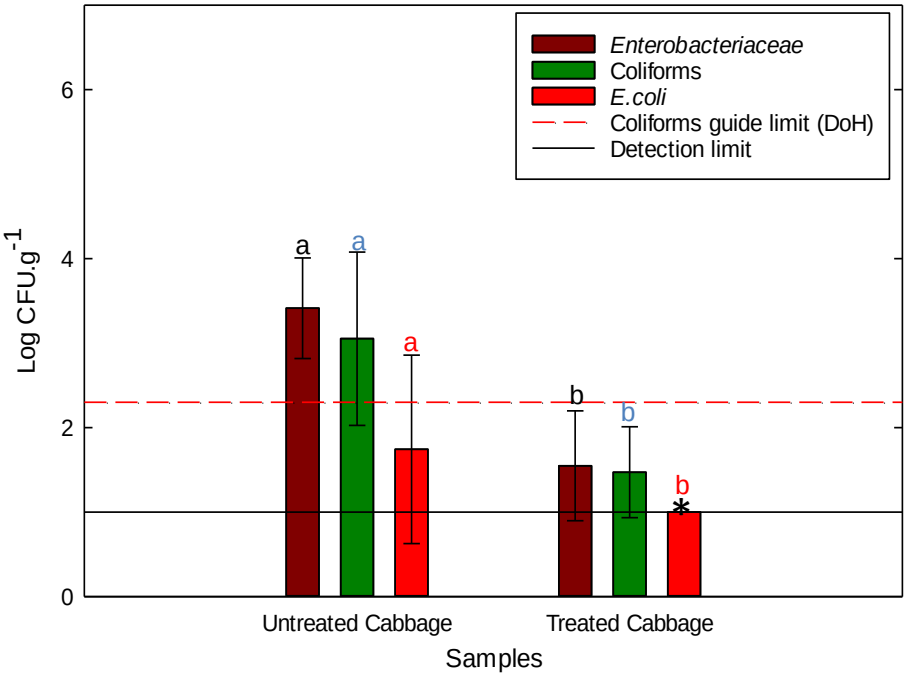

Figure 4.6 Average levels of *Enterobacteriaceae*, coliforms, and *E. coli* on untreated and treated (washed in 150-200 ppm chlorine solution) cabbage samples, and the significant differences. Bars with different letters indicate average counts that are significantly different at 95% confidence level (P<0.05). Bars with the same letters indicate average counts that are not significantly different (P>0.05). Black letters represent *Enterobacteriaceae*; blue letters represent coliforms. ⁎=not detected. The error bars indicate standard deviation. The red dotted line indicates the highest accepted level of coliforms on ready-to-eat fresh produce, by the previous South African department of Health (DoH) (DoH, 2002) (under review). The black line indicates the lowest level at which microorganisms could be detected in this study (1 log CFU.g^{-1}).

Prevalence of microbial indicators (Enterobacteriaceae, coliform, and E. coli) in the mixed coleslaw bags collected from the pack-house and retail outlets

Each coleslaw bag contained a mix of shredded broccoli stem, carrots, and cabbage. The shredded produce was prepared by shredding the treated samples of which the microbial loads are indicated in Figures 4.4, 4.5 and 4.6. It was assumed that the microorganisms recovered from treated broccoli stems (Fig. 4.4), carrot (Fig. 4.5) and cabbage (Fig. 4.6) samples, would be carried over to coleslaw bag samples prepared from the same batch. For the purpose of this study the levels of microorganisms (*Enterobacteriaceae*, coliforms, and *E. coli*) previously detected on treated broccoli, carrot, and cabbage samples from the same batches were thus combined to give theoretical average levels (named “treated combined” in

Fig. 4.7) of microorganism expected to be found on coleslaw samples. This was done by adding the average counts of treated broccoli, carrot and cabbage together, and dividing the sum by three to get the theoretical average counts ("treated combined"). This was done for each population individually e.g. average levels of *Enterobacteriaceae* (2.16 log CFU.g^{-1}) on "treated combined" = average counts of treated broccoli (2.38 log CFU.g^{-1}) + treated carrots (2.55 log CFU.g^{-1}) + treated cabbage 1.55 log CFU.g-1) ÷ by three. According to the results (Fig. 4.7), the theoretical average levels of *Enterobacteriaceae*, coliform and *E. coli* in the mixed coleslaw samples were very low (2.16, 1.97 and 1.11 log CFU.g^{-1} respectively). Coliforms average levels were below the DoH guideline limits (under review) of 2.3 log CFU.g^{-1} (DoH, 2002), and *E. coli* levels were below the EC satisfactory levels of 2 log CFU.g^{-1} (E.C, 2007) for ready-to-eat fresh produce. Ideally, under good hygiene practices and good manufacturing practices, the mixed coleslaw bag samples were expected to have approximately same levels of *Enterobacteriaceae*, coliforms, and *E. coli*, as the theoretical average ("treated combined") counts.

The results presented in Figure 4.7 do, however, indicate that the levels of all three populations recovered from coleslaw bags both on production day (at pack-house) and retail stores two days later were significantly ($P<0.05$) higher than the theoretical values (treated combined). For each of the populations the levels were the following: *Enterobacteriaceae* (5.31 & 6.33 log CFU.g^{-1}), coliforms (5.22 & 6.12 log CFU.g^{-1}) and *E. coli* (2.47 & 2.42 log CFU.g^{-1}) recovered from the coleslaw bags from both the pack-house on the same day and retail outlets two days later, respectively. This could mean that during shredding and packaging, the treated samples might have been contaminated through contaminated workers hands, shredding machine, or packaging materials (Mir *et al.*, 2018).

Van Dyk *et al.* (2016), have reported that coliform levels on tomato samples sourced from the market and processing facility were higher than levels on tomato samples sourced directly from the field. They have concluded that washing and packaging have increased the levels of coliforms. However, in this study, chlorine washing has reduced the level of microorganisms on samples prior to further processing. Therefore, this study does not point towards washing as a reason to the increased levels of microorganisms in the mixed coleslaw bags.

Shredding or packaging could also be contributing factors. A reduction of microorganisms after washing and increase after packaging has been reported in other studies. Zoellner *et al.* (2016), have observed a reduction of total coliforms on tomatoes after washing (<1 log CFU.g^{-1}), which then significantly increased ($p< 0.05$) to 0.7 ± 1.0 log CFU.g^{-1} after sorting and packing.

Increased levels of microorganisms recovered from the mixed coleslaw samples could also be a result of the mixed coleslaw bags being exposed to temperatures favouring the growth of microorganisms. In addition, during shredding, nutrients are released from the produce, becoming available to the existing microorganisms, thereby facilitating the growth of microorganisms (Qadri *et al.*, 2015). Osaili *et al.* (2018) have reported an increase in microbial population numbers in shredded lettuce stored at low temperatures. Under favourable temperatures, an even further increase in microbial levels could be expected.

As mentioned, the microbial levels present in mixed coleslaw bags collected from the pack-house and from retailers were analysed to determine the level of microbial changes observed. The average levels of *Enterobacteriaceae* and coliforms were significantly ($p<0.05$) lower in coleslaw bags sourced from the pack-house directly after packaging than coleslaw bags sourced from the retail two days later. On the other hand, *Escherichia coli* levels recovered from the coleslaw bags collected from the pack-house directly after packaging and those from the retail two days later were not significantly different. The coleslaw samples were all in sealed bags that did not allow extra contamination during transit or handling. The higher microorganism levels in coleslaw bags collected from the retail outlet could be a result of microbial growth over time which could also been influenced by available nutrients and favourable conditions during transportation, or storage or when the bags were on the market shelf.

Several studies have observed a gradual increase in the level of microorganisms on fresh produce throughout the supply chain. Shenge *et al.* (2015), have observed a progressive increase in levels of coliforms and *E. coli* on tomatoes from farm to market, in Nigeria. Zoellner *et al.* (2016) have also reported an increase on levels of total coliforms on tomatoes, along the supply chain (from 0.7±1.0 log $CFU.g^{-1}$ at packing to 1.4 ± 1.5 log $CFU.g^{-1}$ at supermarkets). Conditions to which fresh produce are exposed and time of exposure throughout the supply chain are responsible for changes in levels of microorganisms (Zoellner *et al.*, 2016).

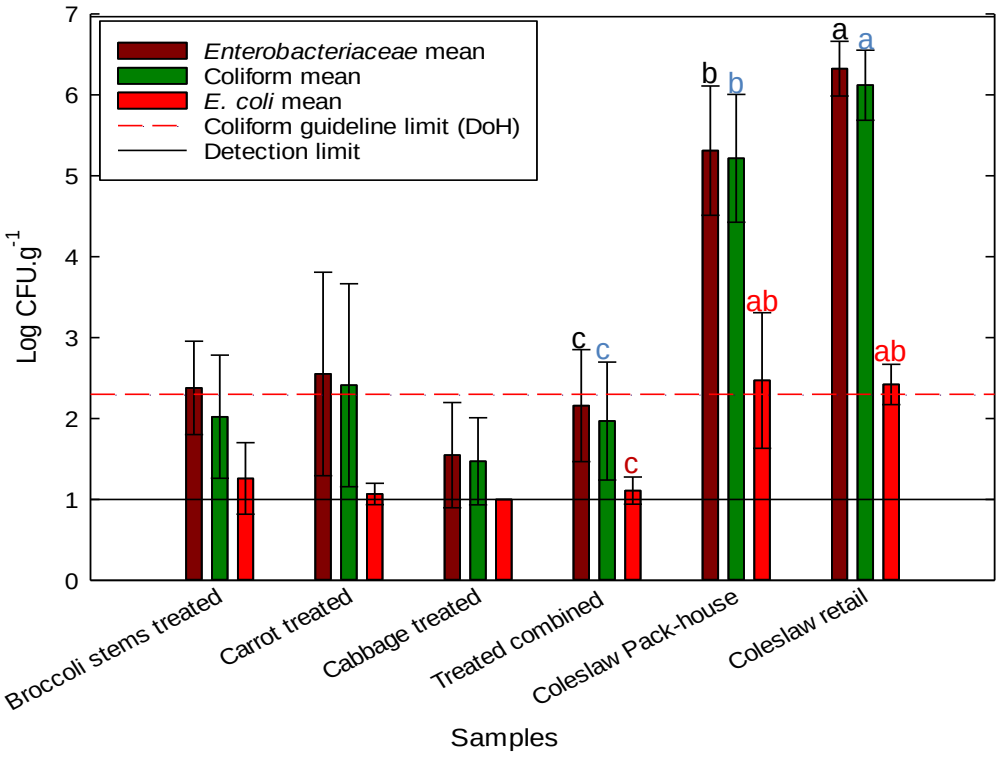

Figure 4.7 Average levels of *Enterobacteriaceae*, coliforms, and *E. coli* on coleslaw bags collected from the pack-house and the retail, and the significant differences. Bars with different letters indicate average counts that are significantly different at 95% confidence level ($p<0.05$). Bars with the same letters indicate average counts that are not significantly different ($p>0.05$). Black letters represent *Enterobacteriaceae*; blue letters represent coliforms. ⁎=not detected. The error bars indicate standard deviation. The red dotted line indicates the highest accepted level of Coliforms on ready-to-eat fresh produce, by the previous South African Department of Health (DoH) (DoH, 2002) (under review). The black line indicates the lowest level at which microorganisms could be detected in this study (1 log CFU^{-1}). Treated = produce washed in 150-200 ppm chlorine solution.

Prevalence of microbial indicators: Enterobacteriaceae, Coliforms and E. coli on lettuce samples

The results for lettuce samples obtained in this Chapter are similar to the lettuce results obtained in Chapter 3. Lettuce head (unprocessed lettuce) carried initial microbial load it acquired from the field and during transportation from the field to the pack-house. Therefore, the average levels of microorganisms recovered from “lettuce head” samples were compared to that of processed lettuce samples to determine the impact of processing on microbial load (Table 4.8). The average levels of *Enterobacteriaceae,* coliforms *and E. coli* recovered from “lettuce head” were 4.7, 4.08 and 2.63 log $CFU.g^{-1}$, respectively (Fig. 4.8). Intact lettuce head was dipped in chlorine solution (150-200 ppm). These washed lettuce heads are referred as “loose lettuce”, as it is sold loose (unpackaged) to the customers. As also previously observed in Chapter 3, lettuce head and loose lettuce samples in this chapter did not show any significant difference in levels of *Enterobacteriaceae* ($p=0.76$) and

coliforms (p=1). *Escherichia coli* were also recovered from loose lettuce. The results (Fig. 4.8 & Table 4.8) indicated no significant difference between the average levels of *E. coli* recovered from the lettuce head and loose lettuce. This indicates that dipping lettuce head in chlorine solution (150-200 ppm) did not significantly reduce microorganisms from lettuce head.

Lettuce was also packaged and supplied to the retail outlets as wrapped whole lettuce (Pre-packs). To determine the microbial changes along the pre-packaged lettuce supply chain, lettuce samples from the same batch were collected from the pack-house (Pre-packs pack-house) as well as the retail outlets (Pre-packs retail). The average level of *Enterobacteriaceae* on Pre-packs samples both from the pack-house and retail outlets was significantly ($p<0.05$) lower than "lettuce head" (Table 4.8). There was no significant difference ($p=0.05$) between the coliform levels recovered from Pre-packs pack-house and "lettuce head" (Figure 4.8 & Table 4.8). The average level of coliforms recovered from Pre-packs from the retail was significantly ($p<0.05$) lower than lettuce head. *Escherichia coli* were also recovered from the pre-packaged lettuce samples, at significantly ($p<0.05$) lower levels than lettuce head.

Table 4. 8 Average levels of *Enterobacteriaceae* (E) and coliforms (C) recovered from different lettuce samples

Samples 1.	**Mean Log CFU.g^{-1}**		**Samples 2.**	**Mean log CFU.g^{-1}**		**Samples 3.**	**Mean log CFU.g^{-1}**	
	E	**C**		**E**	**C**		**E**	**C**
Lettuce head	4.7a	4.08a	**Lettuce head**	4.7a	4.08a	**Lettuce head**	4.7a	4.08 a
Loose lettuce	4.42a	4.13a	**Pre-packs pack-house**	3.61b	3.54b	**Pillow-packs pack-house**	2.27b	2.18 b
			Pre-packs retailer	3.55b	3.03b	**Pillow-packs retention**	2.71b	2.25 b

Values carrying same letter (e.g. 4.08a & 4.13a) indicate insignificant difference on microbial levels between samples, and different letters indicate the significant difference.

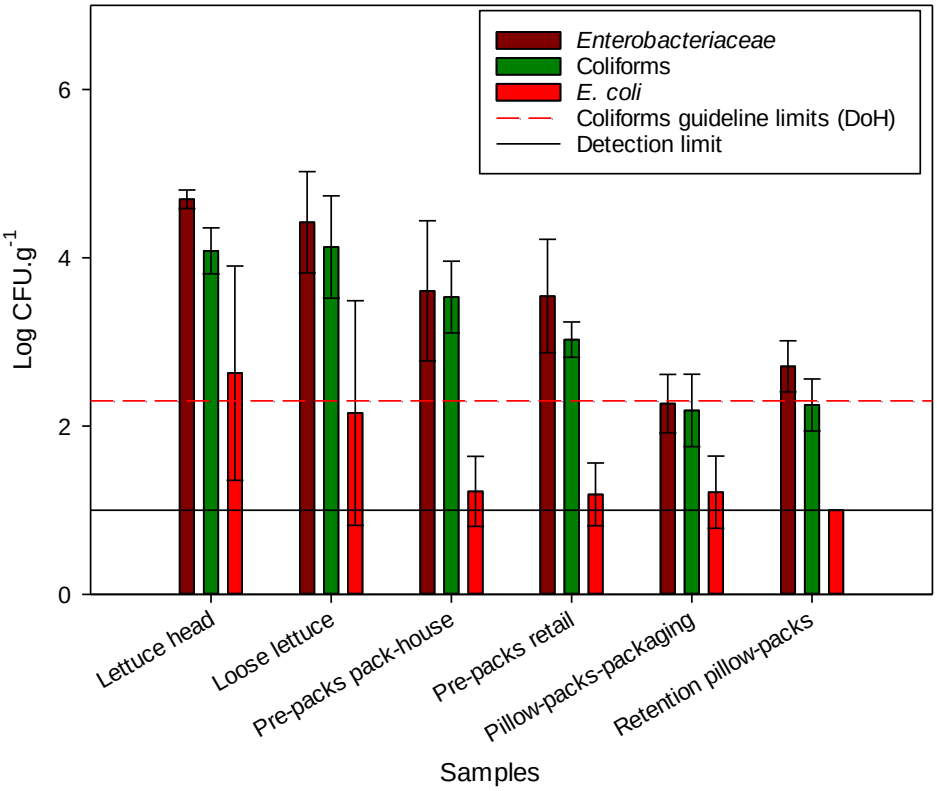

Figure 4.8 Average levels of *Enterobacteriaceae*, coliforms, and *E. coli* on lettuce samples collected from the pack-house and the retailers, and the significant differences. Bars with different letters indicate average counts that are significantly different at 95% confidence level ($p<0.05$). Bars with the same letters indicate average counts that are not significantly different ($p>0.05$). Black letters represent *Enterobacteriaceae*; blue letters represent coliforms. ⁎=not detected. The error bars indicate standard deviation. The red dotted line indicates the highest accepted level of Coliforms on ready-to-eat fresh produce, by the previous South African Department of Health (DoH) (DoH, 2002) (under review). The black line indicates the lowest level at which microorganisms could be detected in this study (1 log CFU^{-1}).

The results (Fig. 4.8 & Table 4.8) indicated no significant difference in the average levels of *Enterobacteriaceae* ($p=0.4$), coliform ($p=1$) and *E. coli* ($p=0.93$) between pre-packs samples collected from the pack-house directly after packaging and at retail outlets two days after. These results indicated a slow decline in levels of microorganisms from lettuce head to pre-packaged lettuce samples. Microbial growth might have been affected by limited nutrients because pre-packaged lettuce was not shredded and could not release extra nutrient. Along the pre-packaged lettuce supply chain, there was no point that could be identified as a potential contamination point.

Lettuce pillow packs were prepared as ready-to-eat bags. As this product was not yet commercially available, all pillow-packs samples were collected from the pack-house. However, three bags were collected on the day of packaging and another three bags (of the same batch) were stored at the pack-house as retention samples to mimic time lapse of retail samples. They were then collected two days later, (on the same day the pre-packaged

lettuce samples were collected from retail outlets). The results (Fig. 4.8 & Table 4.8) obtained indicated a significantly ($p<0.05$) lower average levels of *Enterobacteriaceae* and coliforms in pillow-packs samples than on lettuce head. *Escherichia coli* counts recovered from lettuce head was significantly ($p<0.05$) higher than pillow-packs samples.

The average levels of *Enterobacteriaceae* and coliforms between pillow-packs samples were not significantly different (Fig.4.8 & Table 4.7). However, a slight increase in *Enterobacteriaceae* and coliform levels in retention pillow-packs (sampled two days after production) could be seen (Fig. 4.8). These results indicate that there could be a possibility of further microbial growth should pillow-packs be stored longer. *Escherichia coli* (1.21 log $CFU.g^{-1}$) was only recovered from pillow-packs sampled the day of packaging, and no *E. coli* (< 1 log $CFU.g^{-1}$) was detected on retention pillow-packs samples. Pillow-pack samples had the lowest level of microorganisms, compared to other lettuce samples. Coliform levels recovered from pillow-packs were below the guideline limits (2.3 log $CFU.g^{-1}$) set by the DoH (2002). The previous DoH (2002) (under review) suggests zero *E. coli* level on ready-to-eat fresh produce, while the EC (2007) accept 2 log $CFU.g^{-1}$ of *E. coli* on ready to-eat-fresh produce. Based on the results, the pillow-packs samples in this study were considered as safe for consumption, because levels were below the guideline limits and indicated good microbial quality (International Commission on Microbial Specification of Food (ICMSF), 2001).

Pathogen detection and isolation

Prevalence of STEC on fresh produce

Shiga-toxin producing *E. coli* (STEC) is an important food pathogen associated with diarrheal sickness, which can develop into hemorrhagic colitis (HC) and can eventually results in haemolytic uremic syndrome (HUS) (Baker *et al.*, 2016). STEC causes disease by producing one or more toxins (*stx*1, *stx*2) and it also carries the chromosomal *eae* gene which is responsible for intimate attachment to the intestinal surface (Bryan *et al.*, 2015; Baker *et al.*, 2016). In this study all 72 different samples (lettuce, red cabbage, broccoli stems and carrots) were screened for the presence of STEC. None of the 72 samples tested positive for STEC. However, on two different occasions, *stx* gene and *eae* chromosomal gene were detected separately in two different samples. The *eae* gene was detected in an unwashed lettuce head, and the *stx* gene was found in an untreated carrot sample. However, these two samples could not be described as STEC positive because neither contained all STEC virulence factors (one or two *stx* and the *eae* gene) at the same time. These results are in agreement with results obtained by De Bruin *et al.* (2016) on microbial

quality of fresh basil along the supply chain in Gauteng and Northwest province of South Africa. De Bruin *et al.* (2016) tested the fresh basil for *E. coli* O157:H7 (which is a specific STEC type) from production to the retail outlet, and none of the samples tested positive for *E. coli* O157:H7.

Salmonella

Salmonella spp have been implicated in food-borne outbreaks associated with fresh produce (Jung *et al.*, 2014; Murray *et al.*, 2018). *Salmonella* was detected on fresh-cut organic vegetables in Nigeria by Nguz *et al.* (2005). *Salmonella* in fresh produce can potentially complicate consumer's health. The previous South African DoH (DoH, 2002) (under review) and the EU guidelines (EC, 2007) suggest that *Salmonella* should be absent in ready-to-eat fruits and vegetables. In this study, all 72 samples (lettuce, red cabbage, broccoli stems and carrots) were screened for *Salmonella*. None of these samples tested positive for *Salmonella* spp. Similar results were reported by Van Dyk *et a*l. (2016) who tested commercially produced tomatoes, and found that all sampled tomatoes were free from *Salmonella typhimurium*.

Detection and isolation of ESBL-producing Enterobacteriaceae

A total of 126 different samples (54 lettuce, 18 carrots, 18 red cabbage, 18 broccoli stems, and 18 broccoli coleslaw samples) were screened for ESBL-producing *Enterobacteriaceae* using ChromID Brilliance ESBL agar plates (bioMèriux, South Africa). Of all samples screened for ESBL-producing *Enterobacteriaceae*, 56 produced presumptive positive ESBL-producing colonies. This included 26 lettuce samples and 30 broccoli coleslaw samples (7 broccoli coleslaw, 10 cabbages, 7 carrots, and 6 broccoli stems samples). The results were based on comparison of the colour of colonies observed to the colour chart provided by the manufacturer (*E. coli*: Pink/Burgundy, *Klebsiella/Enterobacter/Serratia*: Blue/green, *Proteus*: light dark). The other 70 of 126 samples did not result in any growth on the ChromID Brilliance ESBL agar (bioMèriux, South Africa).

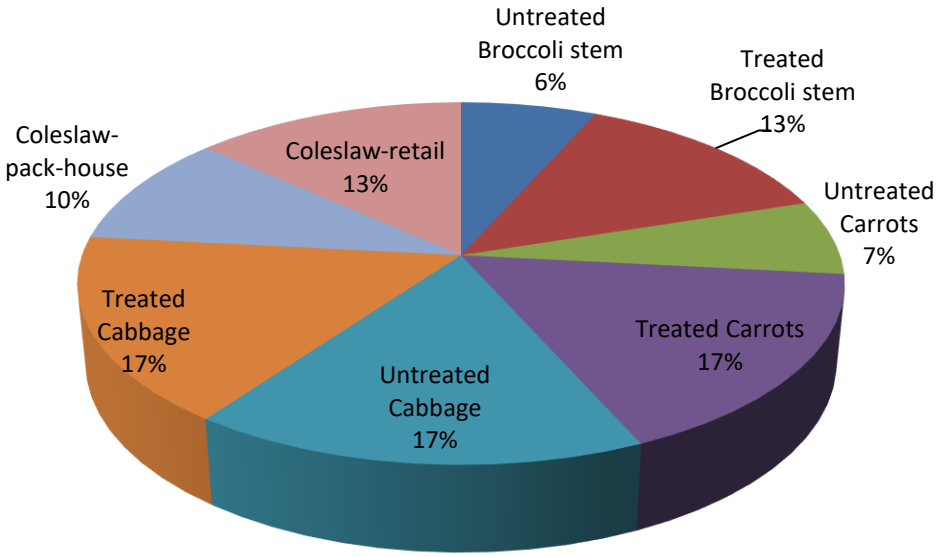

Figure 4. 9 Percentage distributions of 30 coleslaw samples (broccoli stem, carrots, cabbage, and coleslaw bags samples) from which presumptive positive ESBL-producing colonies were isolated

Most presumptive positive ESBL-producing isolates were from untreated cabbage (17%), treated cabbage (17%) and treated carrots (17%). Untreated carrot and untreated broccoli stems samples had the lowest number of positive isolates (7% and 6% respectively) (Fig. 4.9). Some treated samples (treated carrots, treated broccoli coleslaw) had more presumptive positive ESBL-producing isolates than untreated samples. Most positive isolates were expected to come from untreated samples than treated samples. Owing to the fact that untreated samples have been open to possible microbial contaminations while in the field, as well as during handling and transportation. However, the higher occurrence of presumptive ESBL-producing *Enterobacteriaceae* on treated samples compared to untreated samples could be a reflection of new contamination during processing, possibly from surfaces, and workers' hands.

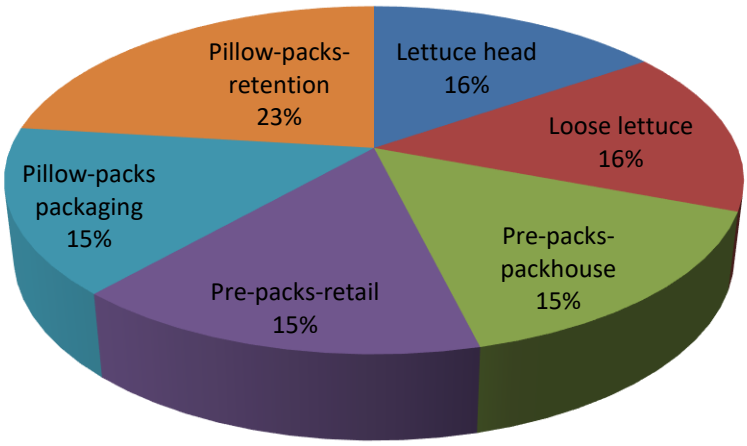

Figure 4.10 Percentage distributions of 26 lettuce samples (lettuce head, loose lettuce, Pre-packs-packs, and pillow-packs samples) from which presumptive positive ESBL-producing colonies were isolated

The suspected isolates were more prevalent in retention pillow-packs (23%) (Fig. 4.10). In this study, retention pillow-packs were found carrying lower levels of *Enterobacteriaceae* and coliforms (2.71 & 2.25 log CFU.g^{-1}, respectively) compared to lettuce head (4.7 & 4.08 CFU.g^{-1}, respectively), loose lettuce (4.42 & 4.13 log CFU.g^{-1}, respectively), and pre-packs (3.61 & 3.54 log CFU.g^{-1}, respectively) (Fig. 4.8 & Table 4.). Therefore, pillow-packs were expected to have the lowest presumptive ESBL-producing *Enterobacteriaceae* isolates. Lettuce head and loose lettuce individually were observed with 16% positive presumptive ESBL-producing organisms. The lowest percentage of isolates (15%) was detected in Pre-packs-retails, Pre-packs-pack-house, and pillow-packs-packaging (Fig.4.10).

Identification of Extended spectrum β-lactamasse producing Enterobacteriaceae

All 56 isolates that were suspected to be ESBL-producing bacteria were identified using MALDI-TOF mass spectrometry. According to the identification results, 50 (89%) of 56 isolates were classified as members of *Enterobacteriaceae* (Table 4.9). Six isolates of the 56 (11 %) were identified as *Pseudomonas spp*, which are non-*Enterobacteriaceae*. The *Enterobacteriaceae* strains indentified were *Enterobacter cloacae* (64%) *Klebsiella oxytoca* (18%), *E. coli* (7%) (Table 4.9). The *Pseudomonas* spp. strains identified were *Pseudomonas* sp [2] (4%), *Pseudomonas putida* (4%) and *Pseudomonas geminis* (4%) (Table 4.9).

Table 4. 9 Identification of fresh produce isolates according to the MALDI-TOF mass spectrometry

Microorganism	Number of isolates	Percentage (%)
Enterobacter cloacae	36	64
Klebsiella oxytoca	10	18
E. coli	4	7
***Pseudomonas* sp [2]**	2	4
Pseudomonas putida	2	4
Pseudomonas geminis	2	4

Antimicrobial susceptibility (ESBL-producing Enterobacteriaceae confirmation)

The *Enterobacteriaceae* strains producing ESBLs are becoming more prevalent in many environments other than clinical environments (Mesa *et al.*, 2006). Several studies done on fresh produce in Gauteng province, South Africa (Richter *et al.*, 2019) and elsewhere (Reuland *et al.*, 2014) have reported the prevalence of *Enterobacteriaceae* strains producing ESBL in fresh produce. In this study, the presence of ESBL-producing *Enterobacteriaceae* in fresh produce samples, and the resistance to antibiotics were investigated. To confirm the ESBL-production, *Enterobacteriaceae*, isolates were subjected to a standard ESBL confirmatory disc diffusion test according to the EUCAST method (2017b). Isolates were confirmed positive for ESBL-production when the growth inhibitory zone diameter around CAZ+CA (30 µg +10 µg) or CTX+CA (30 µg+10 µg) were ≥5 mm larger than the inhibitory zone diameter around CAZ (30 µg) or CTX (30 µg) disc without CA (10 µg) for group 1. For group 2, isolates were confirmed positive when the growth inhibitory zones diameter around CPM+CA (30 µg+10 µg) were ≥5 mm larger than the inhibitory zone diameter around CPM (30 µg) without CA (10 µg) for group 2 (according to the EUCAST 2017b).

Group 1 *Enterobacteriaceae* includes strains like *E. coli*, *Klebsiella* spp., *Salmonella* spp., *Shigella* spp. and *P. mirabilis.* Whereas group 2 *Enterobacteriaceae* consists of organisms containing an inducible chromosomal AmpC like *Enterobacter* spp. Amongst the 50 MALDI-TOF identified *Enterobacteriaceae* isolates in this study, 11 (22%) of them were confirmed as ESBL-producers (Table 4. 10).

All 50 *Enterobacteriaceae* isolates were also tested for resistance against five additional antibiotics representing different classes: ampicillin, gentamicine, tetracycline, ciprofloxacin, and chloramphenicol, as described in the methods section. The results were interpreted according to the CLSI (2016) interpretive criteria (Table 4.4). Most isolates (88%)

were found resistant to Ampicillin, followed by Gentamicine (18%), Chloramphenicol (14%), and Tetracycline (6%). No isolate was found resistant to ciproflaxin, (although 88% of isolates were intermediate and 12% was sensitive to ciprofloxacin).

High resistance (88%) to ampicillin by isolates from fresh produce has been reported in literature. Zurfluh *et al.* (2015) have reported similar results, where all isolate from the produce (100%) was resistant to ampicillin. Similarly, Laubscher (2019) in her study done on fresh produce samples collected from the informal markets in the Western Cape have also found all fresh produce isolates resistant to amplicillin. Ampicillin is a very important antimicrobial frequently used to fight against bacterial infections (Lode, 2008). Isolates that were co-resistant to more than two antimicrobials from different classes were classified as multidrug resistant (Doyle *et al.*, 2013). Out of all 50 *Enterobacteriaceae* isolates only three (10%) isolates were classified as multidrug resistant (Table 4.11). However, these results are limited to the five of antibiotics used in this study. Isolates might also be resistant to other antibiotics classes like sulphonamides, cephamycins, and cephalosporin's, which were not tested in this study.

Table 4. 10 Summary of confirmed ESBL producer strains from fresh produce isolates in this study

			Growth-inhibitory zone diameter (increase)			**ESBL producer (Yes/No)**
Code	**Source**	**Organisms**	**CTX**	**CAZ**	**CPM**	
21A2	Pillow-packs	*Escherichia coli*	26	16	10	Yes
22B	Untreated cabbage	*Enterobacter cloacae*	7	6	5	Yes
22D3	Loose lettuce	*Enterobacter cloacae*	13	6	6	Yes
22D1	Loose lettuce	*Klebsiella oxytoca*	12	7	6	Yes
21E3a	Treated cabbage	*Klebsiella oxytoca*	19	13	10	Yes
21E3b	Treated cabbage	*Klebsiella oxytoca*	19	12	8	Yes
24A1	Pillow-packs	*Klebsiella oxytoca*	20	18	8	Yes
05C1	Treated broccoli stem	*Enterobacter cloacae*	20	13	12	Yes
05C2	Treated broccoli stem	*Enterobacter cloacae*	19	13	12	Yes
05F1	Untreated carrot	*Enterobacter cloacae*	19	13	11	Yes
05F2	Untreated carrot	*Enterobacter cloacae*	21	13	10	Yes

CTX = Cefotaxime, CAZ = Ceftadizime, CPM = Cefepime, treated = washed, untreated = unwashed

Table 4. 11 Antimicrobial susceptibility of ESBL-producing isolates (from fresh produce) to five additional antimicrobials

Code	Source	Organism	Antimicrobial susceptibility					
			Antimicrobial drugs					MDR
			AMP	TE	CIP	GM	C30	Yes/No
ATCC 35218	Reference culture	*Escherichia coli*	R	R	S	S	R	Yes
ATCC 29522	Reference culture	*Escherichia coli*	S	S	I	S	S	No
21A2	Pillow-packs	*Escherichia coli*	R	R	I	R	S	Yes
22B	Untreated cabbage	*Enterobacter cloacae*	R	S	S	S	S	No
22D3	Loose lettuce	*Enterobacter cloacae*	R	S	I	S	S	No
22D1	Loose lettuce	*Klebsiella oxytoca*	R	S	I	S	S	No
21E3a	Treated cabbage	*Klebsiella oxytoca*	R	S	I	I	S	No
21E3b	Treated cabbage	*Klebsiella oxytoca*	R	S	I	R	S	No
24A1	Pillow-packs	*Klebsiella oxytoca*	R	S	I	S	I	No
05C1	Treated broccoli stem	*Enterobacter cloacae*	R	R	I	R	S	Yes
05C2	Treated broccoli stem	*Enterobacter cloacae*	R	R	I	R	S	Yes
05F1	Untreated carrot	*Enterobacter cloacae*	R	R	S	R	S	yes
05F2	Untreated carrot	*Enterobacter cloacae*	R	R	S	R	S	yes

AMP = Amplicillin, TE = Tetracycline, CIP = Ciprofloxacin, GM = Gentamicine, C30 = Chloramphenicol, R = Resistant, S = sensitive, I = Intermediate, treated = washed, untreated = unwashed, MDR = multi-drug resistance

Genotypic confirmation (ESBL genes detection)

It has been reported that *Enterobacteriaceae* strains are increasingly showing resistance to penicillin and the broad-spectrum cephalosporins (Paterson & Bonomo, 2005; Blaak *et al.*, 2014). The resistance to the broad-spectrum cephalosporin results from the production of ESBLs (van Hoek *et al.*, 2015). The most prevalent ESBL genes found in *Enterobacteriaceae* on fresh produce are bla_{TEM}, bla_{SHV}, and bla_{CTX-M} (Reuland *et al.*, 2014; Richter *et al.*, 2019).

In this study, all 50 isolates identified with Maldi-TOF as *Enterobacteriaceae*, (including both confirmed non-ESBL-producers and the confirmed ESBL-producers) were analysed with PCR for genotypic confirmation. The targeted genes were bla_{TEM}, bla_{CTX-M} and bla_{SHV} as indicated in Fig. 4.11. Results indicated that, out of the 50 isolates, only seven isolates were carrying the ESBL genes (Table 4.12). Six of these isolates were co-producers of bla_{CTX} and bla_{TEM}. While one isolate was carrying bla_{TEM} only. The dominant beta-lactamase (*bla*) gene detected in this study was bla_{TEM}, which was detected in seven (14%) of the 50 tested isolates. This was followed by bla_{CTX-M} detected in six (12%) isolates. The gene bla_{SHV} was not detected in any of the tested isolates.

Jena *et al.* (2018) reported similar results, where bla_{TEM} was the most predominant ESBL gene present in 96%, of the tested isolates followed by bla_{CTX-M} with 75%, and bla_{SHV} 18%. However, the strains were isolated from tertiary care hospital, not from fresh produce (Jena *et al.*, 2018). In some studies, bla_{CTX-M} was reported the most predominant β-lactamase gene compared to bla_{TEM} and bla_{SHV}. Ojer-Usoz *et al.* (2014) have found 67% bla_{CTX-M}, 47% bla_{TEM} and 17% bla_{SHV} in waste water treatment plant. Shahid *et al.* (2011) have also found bla_{CTX-M} (29%) dominating in clinical isolates, followed by bla_{SHV} (14%) and bla_{TEM} (11%). Although the genes bla_{CTX-M} and bla_{TEM}, are more common than bla_{SHV} in many studies, the type and predominance of the ESBLs might be influenced by the geographical location (Shahid *et al.,* 2011). The β-lactamase genes are said to be predominant in *Klebsiella pneumonia* and *E. coli* worldwide (Kim *et al.*, 2015; Pitout & Laupland, 2016). In this study, there was no *K. pneumonia* identified. The organisms found carrying bla_{TEM} + bla_{CTX-M} in this study were *Enterobacter cloacae* (50%) and *Klebsiella oxytoca* (50%). A single bla_{TEM} was found in the isolate identified as *E. coli* Table 4.12.

Table 4. 12 A summary of identified organisms with ESBL genes and the sources from which they were isolated

			Disc diffusion	Molecular confirmation
Isolate code	**Source**	**Organism**	**ESBL producer**	**ESBL genes**
21C3	Treated Broccoli stem	*Enterobacter cloacae*	No	TEM + CTX-M
21D1	Treated cabbage	*E. coli*	No	TEM
21E3	Treated carrot	*Klebsiella oxytoca*	Yes	TEM + CTX-M
22B	Untreated cabbage	*Enterobacter cloacae*	Yes	TEM + CTX-M
22D3	Loose lettuce	*Klebsiella oxytoca*	Yes	TEM + CTX-M
22D1	Loose Lettuce	*Klebsiella oxytoca*	Yes	TEM + CTX-M
07bC2	Pre-packs	*Enterobacter cloacae*	No	TEM + CTX-M

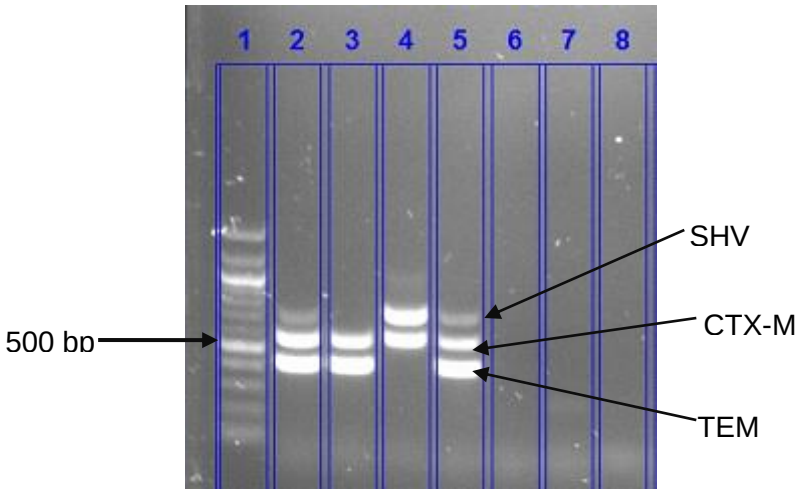

Figure 4.11 Agarose gel (1.2% agarose + 1 µL.10 g^{-1} EZ-Vision blue-light DNA dye) with PCR amplicons including: the positive and negative controls. Lane 1= Ladder, lane 2 & 5 = gene SHV: 747 bp, CTX-M: 445 bp & TEM: 593 bp, lane 3= CTX-M & TEM, lane 4=SHV &CTX-M, lane 6= negative control.

Higher levels of microbial indicators in this study were found in untreated (unwashed & unpeeled samples) samples than in treated (washed in chlorine solution (150-200 ppm) and peeled) samples (Fig. 4.4, 4.5, 4.6 & 4.7). Therefore, it was expected that more isolates from untreated samples would carry β-lactamases genes. However, organisms carrying the β-lactamases genes in this study were mostly identified from treated samples isolates. Only one untreated sample (unwashed cabbage) of the seven isolates was found carrying the ESBL genes in this study. The contaminated soils, irrigation water, surfaces, processing equipment, and animal droppings can be reservoirs of the β-lactamses genes (Blaak *et al.*, 2014). Therefore, the produce may acquire the β-lactamases genes during primary production (from contaminated soils, irrigation water, inadequately composited animal manure, and contaminated harvesting materials), transportation, and during processing (from contaminated surfaces, processing equipment, contaminated wash water, and also from workers) (Muzslay *et al.*, 2017; Freitag *et al.*, 2018).

The fact that the β-lactamase genes were found in isolates from treated samples indicates that ESBL-producing *Enterobacteriaceae* might have been acquired during processing. This is worrisome, because most produce is consumed fresh without heat treatment which may degrade the DNA, and in some cases unwashed. As a result, consumers may acquire *Enterobacteriaceae* carrying the β-lactamase genes. Once ingested, the genes can be transferred to other organisms found in the intestinal tract of humans, causing resistance (Muzslay *et al.*, 2017). The transfer of resistant genes may occur through integrons, which are mobile genetic elements carried within microbial plasmids and transposons (Weldhagen, 2004; Pirzaman & Mojtahedi, 2019). The expressing of β-lactamase genes are known to be responsible for bacterial resistance against the activity of the β-'lactam antibiotics such penicillin, cephalosporin, cephamicins, and carbapenems (Shahid *et al.*, 2011; Pitout & Laupland, 2016). This can result in failure to control infections with the β-lactam family of antibiotics. The β-lactamase genes may interfere with clinical treatment by causing resistance to certain antibiotics like penicillin and cephalosporin (Haghighatpanah *et al.*, 2016).

On the other hand, the organisms carrying these genes may not cause infection in humans, but humans may disseminate β-lactatmases genes in the environment through faecal contaminations (Hölzel *et al.*, 2018).

The ESBL-production ability has been noted in South Africa to be most prevalent in *Klebsiella* and *Enterobacter* spp. (Brink *et al.*, 2006). *Escherichia coli* have also been rapidly developing resistance to many antibiotics used in South Africa (Brink *et al.*, 2006). As indicated in this study, *Klebsiella oxytoca* and *Enterobacter cloacae* strains carried more ESBL genes than *E. coli*. *Klebsiella* species, including *Klebsiella oxytoca,* have been isolated

from fresh produce and other foods, and are also frequently found in clinical samples, (Lowe *et al.*, 2012; Richter *et al.*, 2019). *Klebsiella oxytoca* has emerged as a significant bacterial pathogen resulting in morbidity in humans, by mostly colonising immunocompromised patients and neonates (Lowe *et al.*, 2012). An outbreak of ESBL-producing *Klebsiella oxytoca* has been reported between 2017 and 2018 in special care nursery neonates, however, the source was not identified (Vesey *et al.*, 2018). Lowe *et al.* (2012) have also reported an outbreak associated with ESBL-producing *Klebsiella oxytoca* in Canada's Toronto Hospital (mainly in the "intensive care units, step down units, and medical care units") from 2006 to 2011. Handwashing sinks in the intensive care unit were found contaminated with ESBL-producing *Klebsiella oxytoca* and were indicated as having contributed to the prolonged outbreak (Lowe *et al.*, 2012).

Enterobacter cloacae are found in the gastrointestinal tract of humans and warm blooded animals, and can be transmitted through contaminated environments, surfaces and hands (Bousquet *et al.*, 2017). They cause infections of the urinary tract in humans (Xu & He, 2019). These bacteria have been reported frequently causing nosocomial infections especially in the intensive care unit (ICU) (Van der Mee-Marquet *et al.*, 2006; Bousquet *et al.*, 2017). Fresh produce contaminated with *Klebsiella oxytoca* and *Enterobacter cloacae* strains carrying the ESBL genes can be detrimental to consumer's health.

CONCLUSIONS

The microbial quality of fresh produce (specifically broccoli stem, cabbage, carrot, lettuce and broccoli coleslaw) collected within the pack-house (pre- and post-processing) as well as from the retailers, was successfully evaluated. All samples were tested for microbial indicators (*Enterobacteriaceae*, coliforms and *E. coli*) and the presence of pathogenic *E. coli* (STEC) and *Salmonella*. They were also tested for the presence of antimicrobial resistant strains as well as the presence of ESBL-producing *Enterobacteriacaeae*. The levels of microorganisms on fresh produce collected from different sampling points along the production chain (pack-house to retailer) were evaluated in order to determine microbial changes along the production chain. Untreated samples were found to have significantly higher microbial levels than treated (washed in chlorine solution 150-200 ppm) samples. This is due to the fact that, untreated produce samples were exposed to potential contamination while in the field, during harvest and transportation from the farms to the pack-house. Fresh produce used in this study were treated mainly by washing in chlorine solution (150-200 ppm), however, findings obtained in this study indicated that, the treatment was not effective enough. Microorganisms were not completely removed they were only reduced to certain levels for each produce. The effectiveness of washing produce in chlorine

solutions can be affected by the contact time, pH of the solution or the reaction of chlorine with organic matter from the produce. The latter can form disinfection by-products and reduce chlorine efficacy. In this regard, a comprehensive study assessing the effectiveness of chlorine solution on different produce, as well as factors affecting the disinfection efficacy is recommended.

Microbial levels were significantly higher on the mixed coleslaw samples than any other samples. The coliform average levels found on mixed coleslaw samples were higher than the previous guideline limits set by the DoH (2002) (under review). *Escherichia coli* levels were also above the E.C (2007) guideline limits for ready-to-eat fresh produce. These findings are worrisome because the coleslaw is eaten raw without even further washing, which can expose consumers to microbial populations present on the produce. The growth nutrients released from the shredded produce in combination with favourable temperatures might be the reason for high levels of microorganisms observed in mixed coleslaw samples. On the other hand, mixed coleslaw samples might have been contaminated during shredding and packaging, as a result of contaminated surfaces, packaging materials or workers hands. Therefore, a further study to assess the impact of microorganisms present on workers' hands, packaging material, equipment and surfaces is recommended.

Mixed coleslaw samples collected from the retailers two days after packaging were found with significantly higher average levels of *Enterobacteriaceae* and coliforms than mixed coleslaw bag sampled from the pack-house the day of production. The increase in microbial average levels encountered in the mixed coleslaw samples collected from the retailer might have been induced by the breakdown in the cold chain from the pack-house to retailer point of sale. It is therefore recommended that the exact impact of transport and distribution on microbial numbers be examined in future studies.

In lettuce samples a gradual decrease in the average levels of microorganisms in samples was observed, of which in some samples, like loose lettuce, was not significantly different from the average level of lettuce head (unprocessed lettuce). Pillow-packs samples were observed with the lowest microbial levels compared to "loose lettuce" and pre-packaged lettuce samples. The level of coliforms on pillow-packs was below the previous guideline set by the DoH (2002) (under review), and no *E. coli* was found in pillow-pack samples.

Salmonella and STEC were not detected in any of the produce samples. This does not rule out the possibility that pathogens other than *Salmonella* and STEC might be present. Lettuce pillow-packs samples had more presumptive positive ESBL-producing microorganisms compared to other samples. This might have occurred as a result of post-processing contamination. Twenty percent of the presumptive positive isolates that were

identified as *Enterobacteriacea*e members were confirmed to be ESBL-producers. The genotypic confirmation findings have indicated seven of the tested isolates carried ESBL genes bla_{TEM}, and bla_{CTX-M} only. The bla_{SHV} gene was not found in any of the samples. The findings have also indicated that some isolates (10% of 50 tested isolates) were resistant to multiple antimicrobials (additional antimicrobials). In addition, most Isolates (88%) of the 50 tested isolates were resistant to penicillin. These findings are worrisome because fresh produce is eaten raw, as a result, consumers may acquire resistant bacteria which interfere with treatment against bacterial infections.

Findings obtained in this study gave a limited indication of the microbial quality of some fresh produce sold in the Western Cape in some retailers. However, microbial quality of fresh produce can be different at other pack-houses due to different processing methods used and workers with different understandings about hygiene. It is therefore recommended that, a comprehensive study is conducted that investigate fresh produce quality from different pack-houses in the Western Cape, for more comprehensive findings.

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CHAPTER 5

GENERAL CONCLUSION AND RECOMMENDATIONS

General conclusions

Consumption of fresh produce has become a phenomenon in many countries including South Africa due to its health benefits (Olaimat & Holley, 2012; De Bruin *et al.*, 2016). Unfortunately, fresh produce can get contaminated at any point along the production chain (Francis *et al.*, 2012). Consumption of contaminated fresh produce has been implicated in many food-borne outbreaks (Jung et al., 2014; Wadamori et al., 2017; Murray et al., 2018). Locally, pathogens like *E. coli* O157:H7 and *Salmonella* have been isolated from fresh produce in the Eastern Cape, Gauteng and Northwest provinces in South Africa (Abong *et al.*, 2008; De Bruin *et al.*, 2016). Extended Spectrum β-Lactamase (ESBL) producing *Enterobacteriaceae* family, which confer resistance to broader spectrum cephalosporins' (Pitout & Laupland, 2008; Stadler *et al.*, 2018), is regarded as a significant health threat (Van Hoek *et al.,* 2015). Fresh produce contaminated with resistant strains may transmit the resistant strains to humans through ingestion of this produce (Thanner *et al.*, 2016). Information regarding microbial quality and safety of fresh produce sold in the Western Cape formal markets is however limited

In this study the microbiological quality of fresh produce pre- and post-pack-house processing and at the formal point-of-sale, was successfully evaluated in order to identify potential contamination points along the supply chain. The antimicrobial susceptibility was also successfully evaluated.

The first part of the study (Chapter 3) focused on enumeration of microbial indicators on fresh produce collected before and after pack-house processing steps. The levels of microbial indicators were determined on the following samples: broccoli stems, carrots, and red cabbage collected before processing, after peeling and washing, and after shredding and packaging. Lettuce samples were collected from different processing points of each product that is supplied to retailers or sold to individuals at the pack-house (pre-packs, pillow-packs and loose lettuce). The results showed that, untreated/unprocessed broccoli stems and carrots samples had significantly higher levels of microorganisms than samples that were treated (peeled and washed in chlorine solution (150-200 ppm) for at least a minute). Untreated samples could carry microorganisms from the field or from contact with other contaminated surfaces that occurred during transportation from farms to the pack-house. Peeling and washing the produce in chlorine solution (150-200 ppm) had reduced the

levels of microorganisms to significantly lower levels than in untreated samples. This indicated that washing in chlorine solution (150-200 ppm) might not completely remove microorganisms but can reduce microorganisms to lower levels depending on the initial microbial load on the produce samples. Factors like changes in the pH level, the contact time and reactions of organic matter with chlorine influence the effectiveness of chlorine solution (Murray *et al.*, 2018; Stefán *et al.*, 2019).

Shredding and packaging have been identified as potential contamination points. This observation is based on the significant increase on levels of *Enterobacteriaceae* and coliforms observed on broccoli stems and carrots samples after shredding and packaging. The shredded samples might have been contaminated through workers' hands or contaminated surface. However, the increased levels of microorganisms on shredded produce samples could also imply that, the produce was exposed to temperatures supporting microbial growth, which together with nutrients released from the shredded samples and high-water activity have facilitated the growth of microorganisms. Both shredded broccoli stems, cabbage and carrots samples had coliforms levels exceeding the microbial guideline limit (2.3 log $CFU.g^{-1}$) (under review) set by the South African Department of health (DoH) (DoH, 2012). *Escherichia coli* were not recovered on shredded broccoli stem and cabbage samples. However, it was recovered in shredded carrots samples in levels exceeding the satisfactory levels of ready-to-eat produce described by the European commission (EC, 2007).

Unprocessed lettuce head had higher levels of microorganisms compared to other lettuce samples. However, a gradual decrease in levels of microorganisms has been observed in lettuce samples. Therefore, there was no processing point that could be identified as additional potential contamination point. The lowest microorganisms were found in pillow-packs samples. This could be due to spinning of loose lettuce leaves, which then left the leaves with no available water activity to support microbial growth. Also, lettuce was not shredded to provide microorganisms with growth nutrients released from the produce when shredded/pre-cut. Loose lettuce (washed lettuce head) and pre-packs samples were found with high levels of *Enterobacteriaceae* and coliforms levels (exceeding the DoH guideline limit (under review) of coliforms on ready-to-eat vegetables). However, these products are not sold as ready-to-eat produce; consumers are expected to further process/wash before use. The level of *E. coli* found in lettuce samples throughout, met the EC (2007) satisfactory levels (≤2 log $CFU.g^{-1}$) of *E. coli* on ready-to-eat fresh produce. In summary, the microbiological quality of lettuce samples was satisfactory.

The second part of the study (Chapter 4) focused on the enumeration of microbial indicators, detection of STEC and *Salmonella* and testing for ESBL-producing

Enterobacteriaceae. The produce samples (individual broccoli stems, carrots, red cabbage, coleslaw bags and lettuce samples) were collected from the pack-house before and after processing and some samples (bagged coleslaw and pre-packaged lettuce) were also collected from the retail outlets where they were sold.

Higher levels of *Enterobacteriaceae* and coliforms were recovered from untreated/unprocessed samples than from treated produce samples (peeled and washed in chlorine (150-200 ppm) solution). These organisms were reduced to significantly lower levels after peeling and washing in chlorine (150-200 ppm) solution. For the broccoli coleslaw, the peeled and washed samples (broccoli stems, carrots and cabbage) were shredded, combined and bagged. However, due to possible contaminations assumed to have occurred during shredding and packaging, the bagged coleslaw samples were found with significantly higher levels of microorganisms than the treated samples. Another possible reason for the increase in microbial levels observed in bagged coleslaw mix samples can be the growth of microorganisms that were left on the treated samples, which was induced by growth nutrients released from the shredded produce and temperature favourable for microbial growth.

The results have also given an indication that levels of microorganisms may increase over time. This was demonstrated by higher levels of *Enterobacteriaceae* and coliforms that were detected on bagged coleslaw mix samples collected from the retails two days after packaging, than on bagged coleslaw mix samples collected from the pack-house right after packaging. However, high temperatures during transportation of coleslaw mix bags from the pack-house to the distribution points and on the retail shelves could facilitate microbial growth. The levels of both coliforms and *E. coli* recovered on bagged coleslaw mix samples exceeded the previous DoH guideline limits (under review) for coliforms and *E. coli* for ready-to-eat produce. The *E. coli* levels have also exceeded the EC (2007) satisfactory level (≤2 log CFU.g^{-1}), therefore, these samples are not acceptable, due to poor microbial quality.

The results indicated a gradual decrease in microbial levels in all lettuce samples after washing in chlorine solution (150-200 ppm). The levels of microorganisms recovered from lettuce pre-packs sampled at retailers two days after packaging did not differ from the levels of microorganisms recovered from lettuce pre-packs sampled at the pack-house the day of packaging. Pillow-packs sampled two days after packaging had higher counts than those sampled the day of packaging, however, the differences were not significant. Therefore, this study concluded that microorganisms might grow in pillow-packs with loose leaves over time.

Salmonella and STEC were detected on fresh produce in studies done in other provinces in South Africa. In this study, no *Salmonella* or STEC were detected, therefore, the

samples conform to the EC (2007) and DoH microbiological guideline limit for ready-to-eat fresh produce. Nonetheless, other microorganisms such as *Klebsiella* species and *Enterobacter* species were identified on produce isolates that tested positive for ESBL-producing *Enterobacteriaceae*. Some of these isolates were from treated samples that were prepared for ready-to-eat bags. Therefore, this highlights that regardless the absence of the two pathogens other potential microorganisms can be present.

All produce isolates that were identified as *Enterobacteriaceae* were tested for ESBL-producing organisms. Some of the isolates identified as ESBL producers were from ready-to-eat fresh produce samples. In addition, the majority of the ESBL producers and the multidrug resistant isolates were detected in isolates from treated samples and ready-to-eat samples. Only a few were detected from untreated samples. This could be an indication that the produce was contaminated with ESBL-producing organisms during processing. The other concern is that the ESBL genes (*bla*$_{TEM}$ and *bla*$_{CTX-M}$) were also detected on produce isolates of which some were from the treated (peeled and washed in chlorine (150-200) solution) samples. Treated samples (treated broccoli stem, carrot and cabbage samples) are shredded right after washing, and the shreds are combined and packaged as coleslaw mix. Therefore, organisms carrying the ESBL genes might be carried over to the ready-to-eat mixed coleslaw. Through consumption of contaminated ready-to-eat produce, the ESBL genes might be transferred to humans' intestinal microorganisms. This will then lead to increased risks of antimicrobial resistance, making it difficult for human treatments against pathogenic bacteria, and humans can also disseminate it into the environment.

This study reveals that washing in chlorine (150-200 ppm) solution does not completely remove microorganisms from fresh produce. It has also found that microorganisms raise after shredding and packaging. The study has also found samples collected at the retailers two days after packaging carrying higher levels of microorganisms than samples from same batch collected at the pack-house the day of packaging. Pathogenic microorganisms (*Salmonella* and STEC) were not detected throughout the study. Finally, fresh produce isolates were found carrying ESBL-producing organisms. Through genotypic confirmation, *bla*$_{CTX-M}$ and *bla*$_{TEM}$ genes were found. Some *Enterobacteriaceae* isolates (10%) were found to be multidrug resistant.

Future Studies recommendations

In both studies, fresh produce was washed in chlorine solution (150-200 ppm) for at least a minute, however results obtained indicated that washing did not effectively removed microorganisms from the produce. This led to microorganisms being carried over to the ready-to-eat samples. Therefore, it is important for future studies to look into alternative or

additional treatment methods that may reduce microorganisms on fresh produce to undetectable levels (<1 log CFU.g^{-1}). It will also be helpful to determine the effectiveness of chlorine solution on different produce, as well as factors limiting the disinfection efficacy on fresh produce.

According to the results obtained in this study, it was concluded that shredding and packaging were identified as potential contamination points. Therefore, it might be beneficial for future research to look into factors contributing to contaminations during processing for instance testing the processing equipment, surfaces, and wash water and worker hands.

In the second study, the produce samples that were collected from the retailers two days after packaging were found carrying higher microorganisms than produce samples from the same batch collected from the pack-house the day of packaging. Many factors could have influenced the level of microorganisms in this case. Therefore, it is recommended to examine the exact impact of storage, transport and distribution on microbial numbers in future studies.

Finally, results obtained in this study were limited to the production chain of a single pack-house. However, different pack-houses might have different processing methods and workers who with different levels of understanding of sanitation practices in a fresh produce processing facility. Also, good hygiene maintenance within the processing area may differ between pack-houses. Therefore, there might be a possibility of obtaining different results from different pack-houses. It is for that reason, a more inclusive study is recommended to investigate fresh produce quality along different pack-houses production chain in the Western Cape, for more comprehensive findings.

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